

1 **Mechanochemical feedback and control of endocytosis and membrane tension**

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24 **Abstract**

25 **Plasma membrane tension is an important factor that regulates many key**
26 **cellular processes. Membrane trafficking is tightly coupled to membrane tension**
27 **and can modulate the latter by addition or removal of the membrane. However,**
28 **the cellular pathway(s) involved in these processes are poorly understood. Here**
29 **we find that, among a number of endocytic processes operating simultaneously**
30 **at the cell surface, a dynamin and clathrin-independent pathway, the**
31 **CLIC/GEEC (CG) pathway, is rapidly and specifically upregulated upon**
32 **reduction of tension. On the other hand, inhibition of the CG pathway results in**
33 **lower membrane tension, while up regulation significantly enhances membrane**
34 **tension. We find that vinculin, a well-studied mechanotransducer, mediates the**
35 **tension-dependent regulation of the CG pathway. Vinculin negatively regulates a**
36 **key CG pathway regulator, GBF1, at the plasma membrane in a tension**
37 **dependent manner. Thus, the CG pathway operates in a mechanochemical**
38 **feedback loop with membrane tension potentially leading to homeostatic**
39 **regulation of plasma membrane tension.**

40 **Introduction**

41 Living cells sense and use force for multiple functions like development¹,
42 differentiation², gene expression³, migration⁴ and cancer progression⁵. Cells respond
43 to changes in tension, passively by creating membrane invaginations/ blebs⁶⁻⁸ and
44 actively, by modulating cytoskeletal-membrane connections, mechanosensitive
45 channels and membrane trafficking^{4,9,10}. Membrane trafficking through endo-exocytic
46 processes can respond and modulate the membrane tension¹⁰. While exocytosis acts to
47 reduce plasma membrane tension as a consequence of increasing net membrane area,
48 endocytosis could function to reduce membrane area and enhance membrane tension.

49 Membrane tension has long been shown to affect the endocytic process. Decreasing
50 tension upon stimulated secretion or by addition of amphiphilic compounds increases
51 endocytosis^{11 12}. On the other hand, an increase in tension upon hypotonic shock¹¹ or
52 as evinced during mitosis¹², results in a decrease in endocytosis. Increase in
53 membrane tension on spreading is also compensated via an increase in exocytosis
54 from endocytic recycling compartment providing extra membrane¹³. Together these
55 observations suggest that endocytosis responds to changes in membrane tension or
56 changes in membrane area. However, the specific endocytic mechanisms involved in
57 these responses have not been elucidated. The well-studied dynamin-dependent,
58 clathrin-mediated endocytic (CME) pathway is relatively unaffected by changes in
59 tension¹⁴ while caveolae help to passively buffer membrane tension⁷.

60 We had recently shown that upon relaxing the externally induced strain on cells,
61 tubule like membrane invaginations termed as ‘reservoirs’ are created⁶. This initial
62 response is a purely passive mechanical response of the plasma membrane and is also
63 observed in synthetic lipid membranes. Similar structures are also formed on recovery
64 from hypo-osmotic shock termed as ‘vacuole like dilations’ (VLDs). VLDs form
65 passively, similar to reservoirs, albeit different in shape. Subsequent to the formation
66 of either reservoirs or VLDs, cells engage in an active ATP-dependent response that
67 occurs efficiently only at physiological temperatures to restore their morphology and
68 membrane tension⁶. This indicates the deployment of specific active cellular
69 processes following the passive response (see the cartoon in Fig 1a).

70 Here we have explored the nature of such active responses. We have tested the
71 functioning of multiple endocytic pathways on modulation of membrane tension by
72 different approaches. In parallel, we have determined the effects of modulating
73 endocytic processes on membrane tension by utilizing optical or magnetic tweezers to
74 measure membrane tension. Subsequent to the passive membrane response we had
75 observed earlier⁶, we find that a clathrin, caveolin and dynamin-independent
76 endocytic mechanism, the CLIC/GEEC (CG) pathway, rapidly responds to changes in
77 membrane tension, acting to restore it to a specific set point. Perturbing the CG
78 pathway directly modulates membrane tension forming a negative feedback loop with
79 membrane tension to maintain homeostasis. A previously identified mechanical
80 transducer, vinculin, is involved in the homeostatic control of tension; in its absence
81 the CG pathway fails to respond to changes in membrane tension, thereby altering the
82 set point.

83 **RESULTS:**

84 **A rapid endocytic response to changes in membrane tension**

85 Active cellular processes are involved in resorbing the ‘reservoirs’ or ‘VLDs’ formed
86 following a strain relaxation⁶. To determine whether endocytosis could be one such
87 active process, we monitored the extent of endocytosis by providing a timed pulse of
88 a fluid-phase marker, fluorescent-dextran (F-Dex), during and immediately after the
89 stretch-relax procedure (using a custom built stretch device⁶ shown in Fig. 1b).
90 Compared to cells at steady state, there was a dramatic increase in fluid-phase
91 endocytosis immediately after relaxation of the strain (Fig. 1c) while uptake was
92 markedly reduced during the application of the strain (Supplementary Fig. 1a). This
93 increase in endocytosis was transient and disappeared as early as 90 seconds after
94 strain relaxation (Fig. 1c). This also corresponds to the time scale of resorption of
95 reservoirs by an active process observed earlier⁶. By rapidly upregulating endocytosis,
96 cells thus respond to a net decrease in tension in a fast, transient fashion returning
97 swiftly to a steady state.

98 Earlier studies indicated that exocytosis helped add membrane rapidly in response to
99 increased membrane tension during cell spreading¹⁵. On deadhering, cells round off
100 decreasing their surface area while on replating, cells spread by adding membrane.
101 Thus it is likely that endocytic pathways could help retrieve membrane on deadhering
102 due to decrease in net membrane tension^{8,16}. We reasoned that if an endocytic process
103 is responding to the release of strain during the deadhering process, it would be
104 upregulated during the detachment process. To monitor the extent of endocytosis we
105 followed the uptake of a timed pulse of F-Dex during and immediately after the
106 detachment (3 minutes) and compared it to that measured in the spread state (Fig. 1d
107 schematic). Our results showed that the net fluid-phase uptake underwent a rapid
108 increase while the cells were de-adhering (3 minutes), but subsided back to the steady
109 state level once it was de-adhered and held in suspension (Fig. 1d). Recycling of the
110 endocytic material is similar between steady state and deadhering (Supplementary
111 Fig. 1b). This indicates that there is no block in the recycling rate during deadhering
112 and increase in uptake on deadhering is due to a transient increase in endocytic
113 potential.

114 To further consolidate our findings, we used an alternate method to alter membrane
115 tension. We shifted cells from hypotonic to isotonic medium, which made passive

116 invaginations similar to reservoirs called VLD's⁶. This method also results in an
117 enhancement of fluid-phase endocytosis (Supplementary Fig. 1c), consistent with the
118 results obtained by the other two methods of altering membrane tension. Together
119 these results suggest that reduction of membrane tension via a number of different
120 methods triggered a fast and transient endocytic response on the time scale of
121 seconds.

122 **Membrane tension and the response of multiple endocytic pathways**

123 To ascertain which of the multiple endocytic pathways respond to changes in tension,
124 we examined cargo previously shown to be endocytosed via these distinct pathways.
125 A number of endocytic pathways function concurrently at the cell surface¹⁷⁻²⁰. In
126 addition to the well characterized CME pathway, there are pathways that are
127 independent of clathrin but utilize dynamin for vesicle pinching^{19,21}. Additionally,
128 there are clathrin and dynamin independent pathways which function in a number of
129 cell lines²²⁻²⁴, but not in all²⁵. The CLIC/GEEC (clathrin independent carrier/ GPI-
130 anchored protein enriched early endosomal compartment) pathway is a clathrin and
131 dynamin-independent pathway, responsible for the internalization of a major fraction
132 of the fluid-phase and several GPI-anchored proteins (GPI-AP)^{22,24}, and other plasma
133 membrane proteins such as CD44²⁶. Therefore, we used these specific cargoes to test
134 the response of different endocytic pathways while membrane tension was altered.

135 The endocytic uptake of the transferrin receptor (TfR), a marker of CME, did not
136 increase in the cells which exhibited a transient rise in the fluid-phase after a
137 hypotonic shock (Fig 2a) or detachment (Fig. 2b) as visualized using two color
138 fluorescence microscopy. However, uptake of the folate receptor, a GPI-AP that is
139 internalized via the CG pathway^{27,28}, exhibited a considerable increase (Fig. 2c). This
140 indicated that clathrin-independent endocytosis rather than CME might be involved in
141 the fast response to a decrease in membrane tension.

142 There are endocytic pathways which utilize dynamin independent of clathrin
143 function^{19,22}. Therefore we tested whether the increase in fluid-phase uptake requires
144 dynamin function. We used a conditional triple knock out cell line that removes
145 Dynamin 1, 2 and 3 from the genome²⁹, thereby abolishing all the dynamin-mediated
146 endocytic pathways (Supplementary Fig. 2a). The dynamin triple knockout mouse
147 embryonic fibroblasts (MEFs) shows higher steady state fluid-phase endocytosis²⁹.
148 However, cells lacking all forms of dynamin also transiently increased their fluid-
149 phase endocytosis upon both stretch-relax cycles to the same extent as wild type
150 (WT)-MEFs (Fig. 3a) and hypotonic/isotonic media changes (Supplementary Fig. 2b).
151 Thus, neither CME nor dynamin-dependent endocytic pathways appear to respond to
152 an acute reduction in membrane tension.

153 A caveolin-dependent endocytic process is important to retrieve specialized
154 membrane on deadhering³⁰, and a caveolae-mediated passive mechanism is reported
155 to buffer the increase in membrane tension and prevent cell lysis triggered by the
156 flattening of caveolae⁷. To test if caveolin-dependent endocytic mechanisms could be
157 important for this rapid endocytic up-regulation, caveolin null MEFs were subjected
158 to the stretch-relax protocol. These cells exhibited a transient increase in fluid-phase
159 uptake similar to their WT controls (Fig. 3a). In addition, caveolin-null cells also
160 exhibit a fast transient upregulation of fluid-phase endocytosis during de-adhering as
161 well (Supplementary Fig. 2c).

162 We next examined the morphology of the endocytic carriers formed by reduction of
163 membrane tension induced by deadhoring using electron microscopy (EM). For this,
164 we utilized Cholera Toxin bound HRP (CTxBHRP), which marks the internalized
165 plasma membrane. We used a procedure in which the surface remnant peroxidase
166 reaction product is quenched with ascorbic acid, revealing only the internalized
167 CTxBHRP labeled membrane²⁶. After 5 minutes post-deadhoring the major endocytic
168 structures labeled had the typical morphology of CG carriers (or CLICs) comprising
169 structures with tubular and ring-shaped morphology (arrows, Supplementary Fig. 2d).
170 Morphologically-identical structures were also observed in WT MEFs at steady
171 state³¹ and in Cav1^{-/-} MEFs (arrows, Supplementary Fig. 2d) consistent with the
172 observation of fast fluid-phase uptake in Cav1^{-/-} cells via CG (Fig. 3a and
173 Supplementary Fig. 2c). At this time point, surface-connected caveolae (containing no
174 peroxidase-reaction product) persist in the Cav-expressing WT cells (arrowheads,
175 Supplementary Fig. 2d), consistent with the possibility that the caveolar pathway does
176 not play a significant role in transiently modulating endocytosis at these early times of
177 deadhoring.

178 Together, these experiments indicated that the clathrin, dynamin or caveolin
179 dependent endocytic mechanisms do not exhibit a rapid respond to a reduction in
180 membrane tension. This is in contrast to fluid-phase or GPI-anchored protein uptake
181 which is endocytosed via the CG pathway. CG-mediated endocytosis is a high
182 capacity pathway capable of internalizing the equivalent of the entire plasma
183 membrane area in 12 minutes²⁶, and of recycling a large fraction of endocytosed
184 material³². This pathway is also implicated in the delivery of membrane on cell
185 spreading in response to increases in membrane tension, thus helping to maintain
186 membrane homeostasis^{9,13}.

187 **CLIC/GEEC (CG) pathway responds to membrane tension**

188 Since the CG cargo responded to changes in tension, we explored this finding in
189 further detail. CG pathway is regulated by small GTPase's, ARF1, its GEF GBF1, and
190 CDC42 at the plasma membrane^{28,33,34}. Hence, we utilized small molecule inhibitors
191 of CDC42 and GBF1 to acutely inhibit CG pathway^{35,36}. The CDC42 inhibitor,
192 ML141 decreases fluid-phase endocytosis in cells at steady state but not CME
193 (Supplementary Fig 3a), and prevents the increase in fluid-phase uptake upon
194 deadhoring (Supplementary Fig. 3b). In separate experiments, we utilized LG186, an
195 inhibitor of GBF1, which also decreases fluid-phase endocytosis in cells at steady
196 state but does not affect CME (Supplementary Fig 3c). Inhibiting GBF1 prevents the
197 increase in fluid-phase endocytosis observed upon stretch-relax (Fig. 3b) or
198 deadhoring (Supplementary Fig. 3d). Similar to the decrease in fluid-phase on
199 increasing tension during stretch (Supplementary Fig. 1a), CD44, a CG pathway
200 specific cargo, shows reduced endocytosis during hypotonic shock (Supplementary
201 Fig. 3e).

202 To further confirm that this response is due to CG endocytosis, we assessed the effect
203 of the stretch-relax protocol on cells that lack CG endocytosis. HeLa cells have been
204 shown to lack a robust CG endocytic pathway^{25,27}. While the molecular basis for this
205 defect is not understood, we find that fluid-phase endocytosis in HeLa cells is not
206 susceptible to GBF1 inhibition by LG186 (Supplementary Fig. 4a). In addition, these
207 cells do not show an obvious recruitment of GBF1 to the plasma membrane in the
208 form of punctae as observed in cells exhibiting constitutive CG endocytosis such as

209 CHO cells as reported earlier³⁴ (Supplementary Fig 4b/4c). Correspondingly, these
210 cells did not exhibit a rapid increase in fluid-phase endocytosis on a hypotonic to
211 isotonic shift (Supplementary Fig. 4d).

212 These experiments, combined with the up-regulated endocytosis of a CG specific
213 cargo (GPI-AP) (Fig. 2c), suggest that the CG endocytic pathway is specifically
214 involved in the rapid, transient response to changes in membrane tension.

215 **Passive and active membrane response to changes in membrane tension**

216 As mentioned above, upon a rapid reduction in membrane tension, cells form passive
217 structures such as reservoirs and VLDs similar to the response of an artificial
218 membrane. Reservoirs are formed upon strain relaxation in the membrane after
219 stretching cells, whereas VLDs are formed by water expelled by the cell after a hypo-
220 to-isotonic-shock recovery⁶. Both reservoirs and VLDs are reabsorbed and disappear
221 within a couple of minutes, coincidental with an increase in endocytosis. This led us
222 to test if inhibiting the CG pathway could have a measurable impact on the rate of
223 disappearance of such passive structures. We find that the CG pathway exhibits
224 exquisite temperature sensitivity and is barely functional at room temperature (RT),
225 and is not efficient even at 30°C in comparison to CME in CHO cells (Fig. 4a).
226 Correspondingly, the reservoir resorption in CHO cells was impaired after lowering
227 temperature (Fig. 4b). In addition, inhibition of the CG pathway in CHO cells by
228 inhibiting GBF1 reduced the rate of reservoir reabsorption at 37 °C (Fig. 4a). In
229 contrast, HeLa cells lacking a characteristic CG pathway did not show any difference
230 in the rate of disappearance of reservoirs upon inhibiting GBF1 and was much less
231 affected by lowering of temperature than CHO cells (Supplementary Fig. 4e). Thus,
232 perturbing CG endocytosis affects the kinetics of resorption of reservoirs.

233 We next examined if passively generated structures could help initiate endocytosis at
234 the sites of their formation. Since the disappearance of each reservoir is gradual and
235 not as a single step process⁶ (Fig. 4b), this indicates that reservoirs are not likely to be
236 pinched off directly as endosomes. Further, we do not observe endosomes form at the
237 site of the reservoirs (Supplementary Fig. 5a). To test this, we took advantage of our
238 earlier observation that cells plated on polyacrylamide gels do not form VLDs upon
239 hypotonic to isotonic shifts⁶. Whereas the lack of generation of VLDs was confirmed
240 in our cells grown on polyacrylamide (Supplementary Fig. 5b), the cells still showed
241 an increase in endocytosis similar to when plated on glass, upon exposure to hypo-to-
242 isotonic-shock procedure (Supplementary Fig. 5c). Together, these data suggest that
243 CG endocytosis occurs subsequent to the passive responses of the membrane but the
244 passive invagination formation is not necessary to form CG endosomes. However, the
245 transient increase in CG endocytosis following the passive response helps swiftly
246 resorb the excess membrane helping to restore the membrane morphology.

247 **Role of the CG pathway in setting membrane tension**

248 Since the CG endocytic pathway responded to changes in membrane tension we
249 hypothesized that it might be involved in the setting of steady state membrane tension
250 as well. To explore this hypothesis, we directly measured tether forces by pulling
251 membrane tethers using optical tweezers³⁷. The force experienced by membrane
252 tethers provides a way to measure the effective membrane tension³⁸ (Fig 5a). We
253 found that acutely inhibiting the CG pathway by inhibiting GBF1 drastically reduced

254 the tether forces in a resting cell (Fig. 5b). To further assess this, we applied 0.5 nN
255 force pulses using a magnetic tweezer device to ConcanavalinA (ConA)-coated
256 magnetic beads attached to the cell membrane (Supplementary Fig 5d). Consistent
257 with optical tweezers results, resistance to force (stiffness) was reduced in GBF1
258 inhibited cells (Supplementary Fig 5e). That this effect was due to a reduction in
259 membrane tension and not any effects on the cytoskeleton, was corroborated by the
260 lack of a change in the measured stiffness of fibronectin-coated beads attached to cells
261 via integrin-fibronectin adhesions with and without GBF1 inhibition (Supplementary
262 Fig 5f).

263 We next examined tether forces in cells wherein the CG pathway is up-regulated. We
264 reasoned that since the Dynamin TKO cells show a higher fluid-phase endocytosis
265 (Fig. 5c, Supplementary Fig 6c), it is likely that this would increase effective
266 membrane tension. Tether forces were indeed higher in the Dynamin TKO cells
267 compared to control cells (Fig. 5d). Consistent with the role of the CG-pathway in
268 setting membrane tension, inhibiting the CG pathway in Dynamin TKO cells by
269 GBF1 inhibition (Fig. 5c, Supplementary Fig 5h) reduced the effective membrane
270 tension below control levels (Fig. 5d).

271 To further confirm this observation, we measured tether forces on acutely increasing
272 CG endocytosis by using BrefeldinA(BFA) as reported earlier³³. BFA treatment
273 disrupts ER to Golgi secretion but also serves to free up ARF1, making it available at
274 the cell surface to increase CG endocytosis³³. We further confirm that this increase is
275 mediated through a GBF1-sensitive CG endocytosis (Fig 5e). We treated the cells
276 with BFA and measured tether forces using optical tweezers when the increase in
277 endocytosis was most prominent. Tether forces were higher on treating cells with
278 BFA compared to the control case (Fig. 5f). BFA treatment inhibits secretion³⁹ and
279 this could also increase the effective membrane tension due to a reduction of
280 membrane delivery from the secretory pathway independent of its effect on CG
281 endocytosis. To test this, we treated HeLa cells with BFA. BFA treatment disrupted
282 the Golgi in both CHO and HeLa cells (Supplementary Fig. 5g) consistent with its
283 inhibition of the secretory pathway. However, neither fluid-phase uptake (Fig. 5e) nor
284 the tether forces were affected in HeLa cells (Fig. 5f). This indicated that the increase
285 in tension in CHO cells on BFA treatment is due to an increase in CG endocytosis and
286 not due to a block in secretion in these timescales.

287 Hence, modulating the CG pathway by activating or inhibiting key regulators
288 modifies effective membrane tension directly. Since CG pathway is negatively
289 regulated by membrane tension, this indicates that CG pathway operates in a negative
290 feedback loop with membrane tension. Since CG pathway is specifically modulated
291 by tension, it is conceivable that the molecular machinery regulating CG pathway
292 would be modulated by changes in tension.

293 **Mechanical manipulation of the CG endocytosis machinery**

294 We tested if key regulatory molecules involved in different endocytic pathways could
295 be directly modulated by changes in tension. GBF1 is involved in the CG pathway
296 and re-localizes from the cytosol to distinct punctae at the plasma membrane upon
297 activation as visualized using TIRF microscopy^{33,34}. We imaged GBF1-GFP
298 recruitment to the plasma membrane in live cells using TIRF microscopy, during a
299 hypotonic shock and after recovering from it. GBF1 punctae were lost on hypotonic

300 shock (Fig 6a and 6b) indicating a direct response by GBF1 on increasing tension. On
301 the other hand, recovery from a hypotonic shock caused the rapid assembly of GBF1
302 punctae (Fig 6a and Fig 6b). In contrast, clathrin, which is localized from cytosol to
303 membrane to help in CME is not affected by these similar changes in tension
304 (Supplementary Fig 6a). These experiments indicated that molecular machinery
305 involved in regulating the CG pathway was modulated by membrane tension unlike
306 that of the CME pathway.

307 **Vinculin serves as a mechanotransducer for CG endocytosis**

308 For cells to respond to changes in tension, sensing and transduction of this
309 information must occur. Since focal adhesion related molecules help transduce and
310 respond to force^{5,40,41} we hypothesized these molecules could transduce a physical
311 stimuli (membrane tension) to regulate endocytic processes as well. Indeed, few of
312 these proteins were ‘hits’ in a recent RNAi screen for genes that influence CG
313 endocytosis⁴². Focal adhesion is an intricate macromolecular complex that has
314 multiple functional modules⁴³ and vinculin is a critical part of this
315 mechanotransduction machinery^{40,41,43}. Unlike the ‘hits’, Talin or p130CAS, there is
316 only a single functional isoform of vinculin in non-muscle cells, and cell lines
317 deficient for this protein are viable. Therefore we used a vinculin null fibroblast cell
318 line to test the role of this mechanotransducer in the mechano-responsive behavior of
319 CG pathway.

320 To directly test if vinculin could be involved in the tension-sensitive regulation of the
321 CG pathway, we stretched vinculin null cells. Unlike WT MEFs that shows ~82%
322 drop in uptake on stretching (Supplementary Fig 1a), vinculin null cells show only
323 ~36% drop at the same strain (Fig 7a). Increasing the extent of hypotonic shock
324 showed a concomitant decrease in fluid-phase endocytosis of WT cells (Fig 7b). By
325 contrast, vinculin null MEFs were much more refractory to the same extent of
326 hypotonic shock (Fig 7b). Furthermore, upon strain-relaxation, fluid-phase
327 endocytosis in vinculin null MEFs did not show an increase (Fig 7a), unlike that
328 observed for CHO cells (Fig 1c) or wild type MEFs (Fig 3a). This was further tested
329 in the deadhhering assay where vinculin null cells again did not show an increased
330 fluid-phase uptake, unlike the WT control cells (Supplementary Fig 6c). Thus
331 vinculin null cells do not respond to changes in tension similar to the WT cells.

332 Fluid-phase uptake in vinculin null MEFs is much higher than wild type cells (Fig
333 7c). To test if the endocytic effects of vinculin null cells are specifically due to
334 vinculin, we expressed full length vinculin in vinculin null cells. This caused a
335 decrease in fluid-phase endocytosis (Supplementary Fig 6d). Further, inhibiting GBF1
336 in vinculin-null cells with LG186 decreased fluid-phase uptake to the same levels as
337 cells expressing vinculin, confirming that a GBF1 sensitive CG pathway is functional
338 here (Supplementary Fig 6d). This indicates that GBF1 operates downstream of
339 vinculin and vinculin negatively regulates CG pathway.

340 Since vinculin-null cells have a higher basal endocytosis rate it is possible that they
341 are unable to increase their endocytic capacity further in response to a decrease in
342 membrane tension. However, vinculin null cells respond to BFA treatment to increase
343 their endocytic rate in a manner that is also sensitive to GBF1 inhibition, similar to
344 wild type cells (Supplementary Fig 7a).

345 We next tested if GBF1 shows a tension-dependent membrane localization of GBF1
346 in vinculin null cells. The level of punctae remained constant and failed to respond to
347 hypotonic shock (Fig 7d) unlike that observed in WT MEF (Fig 6a). The density of
348 GBF1 punctae at the plasma membrane was also slightly higher in vinculin null cells
349 compared to WT cells (Supplementary Fig 6b). This is consistent with higher fluid-
350 phase endocytosis in vinculin null cells compared to control MEF cell line (Fig 7c).

351 Further, we tested how the steady state membrane tension in vinculin null cells is
352 compared to WT cells. Tether forces measured using optical tweezers showed a
353 higher value for vinculin null cells compared to wild type cells (Fig 8a). The high
354 tether force in cells lacking vinculin was drastically reduced on inhibiting the CG
355 pathway (Fig 8a), consistent with the role of the CG pathway in regulating the
356 effective membrane tension. These experiments show that vinculin acts as a negative
357 regulator of CG pathway and is necessary for the transduction of physical stimuli for
358 the biochemical control of the CG pathway.

359 DISCUSSION

360 Membrane tension has been long proposed to be tightly coupled to vesicular
361 trafficking through endo-exocytic pathways. However, the specific trafficking
362 mechanisms have remained elusive. Here, we show that membrane tension and CG
363 endocytosis operate in a negative feedback loop that helps restore any change from a
364 set point (model: Supplementary Fig. 8b). When membrane tension decreases, it
365 transiently triggers the CG pathway, bringing about a fast endocytic response to reset
366 the cell's resting membrane tension. On the other hand, increasing membrane tension
367 has the opposite effect; the CG endocytosis is inhibited in a proportional manner. The
368 membrane flux through the CG pathway also has an effect on the effective membrane
369 tension. Acutely lowering the CG pathway decreases membrane tension while
370 upregulating the pathway increases membrane tension. Thus, changes in membrane
371 tension lead to an inverse effect on CG endocytosis, while changes in CG endocytosis
372 lead to a direct effect on plasma membrane tension. This type of a response known as
373 a negative feedback loop is used in many different biological contexts to maintain
374 homeostasis⁴⁴.

375 The CG endocytic pathway specifically responds to acute changes in membrane
376 tension despite multiple pathways operating simultaneously at the plasma membrane.
377 Caveolae passively buffer increases in tension⁷, while the clathrin-mediated pathway
378 concentrates specific ligands and mediates robust endocytosis despite the increase in
379 tension¹⁴. De-adhered cells exhibit an increased caveolin-mediated internalization that
380 persists over hours, and is crucial for the removal of specific membrane constituents
381 and anchorage-dependent growth and anoikis³⁰. On the other hand, unlike the
382 caveolar pathway, the CG pathway showed a higher transient upregulation of
383 endocytosis only during deadhoring which does not persist in suspension.

384 The fast response of the CG pathway on strain relaxation is lost within 90 seconds.
385 The loss of this transient response could be even faster as at present 90 seconds is the
386 dead time in our experiments. Fast clathrin-independent mechanisms have been
387 reported in different contexts. Ultrafast endocytosis occurs at synapses following a
388 synaptic vesicle fusion to retrieve the excess membrane. This fast clathrin-
389 independent but dynamin-dependent process is temperature sensitive as well^{45,46}.
390 Endophilin dependent FEME pathway is another clathrin-independent but dynamin-

391 dependent pathway. HeLa cells seem to predominantly have AP2, GRAF1 and
392 dynamin-dependent machinery²⁵. Consistent with this, inhibiting AP2 or GRAF1 in
393 HeLa cells inhibits fluid-phase endocytosis. These cells respond in a slower rate to the
394 changes in osmotic shock and shows blebbing on inhibiting this pathway due to lack
395 of endocytosis⁴⁷. Here, we find that the GBF1/ARF1/CDC42 dependent CG pathway
396 shows a fast transient response to changes in tension and is more sensitive to lowering
397 of physiological temperature compared to the CME pathway. In the absence of such a
398 fast pathway, other slower endocytic mechanisms operate to internalize the excess
399 membrane, lack of which might lead to blebbing. The CG pathway helps to swiftly
400 respond and reset any changes from the steady state, thereby also helping to set the
401 resting membrane tension of a cell. This indicates that different endocytic pathways
402 have distinct functions and the CG pathway may be responsible for membrane tension
403 homeostasis.

404 Similar to the endocytic response, exocytic processes in a cell could modulate and
405 respond to changes in tension. Exocytic processes in a cell help in addition of
406 membrane to the cell surface and reduction of membrane tension⁹. Unlike
407 endocytosis, exocytosis seems to be positively regulated by membrane tension. High
408 membrane tension increases the exocytic rate and could regulate the mechanism of
409 vesicle fusion^{48,49}. Increase in membrane tension during cell spreading activates
410 exocytosis to increase spread area through a GPI-anchored protein rich endocytic
411 recycling compartment^{13,15}. This increase in area is independent of secretory pathway
412 or other exocytic mechanisms. CG pathway takes in a major fraction of GPI-anchored
413 proteins²⁷ and recycles a huge fraction of its endocytic volume³². On increasing
414 tension, we find that CG endocytosis is downregulated preventing further increase in
415 tension but it could be recycling through CG pathway that helps add membrane to
416 restore the steady state tension (Cartoon: Fig 8b). Thus, regulation of membrane
417 cycling through the endo-exocytic leg of CG pathway could be important in
418 membrane homeostasis and further research would help bring about a complete view
419 of this mechanism.

420 We find that the active response by CG pathway follows the passive response via
421 membrane invaginations (i.e. reservoirs and VLD) and helps in efficient resorption of
422 these passive local membrane structures. There could be other active cellular
423 mechanisms driving the flattening of these invaginations as well. However, these
424 membrane invaginations are not necessary for the creation of the CG endosomes.
425 Thus, following a reduction in tension these are two parallel responses by the cell, one
426 passive and the other active eventually leading to excess membrane internalization
427 through CG endosomes.

428 Similar to the passive response, physical parameters could directly regulate the active
429 endocytic machinery by influencing the extent of membrane deformation needed to
430 make an endocytic vesicle. A higher membrane tension makes it more difficult to
431 deform the membrane, thus producing fewer endosomes, and vice versa, alleviating
432 the need for a specific mechanotransduction machinery. However, our results from
433 studying the vinculin-null cells suggest otherwise. Vinculin, a key focal adhesion
434 protein, transduces many mechanical inputs at the site of the focal adhesion into
435 information for the cell to process^{41,43,50}. In this context, it appears that vinculin plays
436 a central role in transducing the increase (or decrease) in membrane tension to the CG
437 pathway to help inhibit (or activate) its endocytic mechanism (Cartoon: Fig 8b). This

438 appears to be effected by its control of a key regulator of CG endocytosis, GBF1, the
439 GEF for ARF1. In WT cells, GBF1 forms tension-sensitive punctae at the cell surface
440 wherein increasing tension abolishes these punctae and decreasing tension increases
441 it. In the vinculin-null cells, this tension-dependent regulation is lost and CG
442 endocytosis appears to be uncoupled from tension regulation. Thus, vinculin is
443 important for a tension-sensitive negative regulation of a key effector of CG pathway,
444 translating mechanical information into a biochemical read out to influence the
445 endocytic rate. This negative feedback loop between effective membrane tension and
446 CG pathway thus is mediated through vinculin and maintains the cells at a lower
447 effective membrane tension. Different functional modules operate in a focal adhesion
448 for mechanotransduction⁴³. However, the precise mechanism behind the ability for
449 vinculin to regulate the availability of GBF1 at the cell surface is not yet understood
450 and is a subject of further investigation.

451 Modulations in membrane tension are used in multiple cellular processes^{4,51-54} and
452 CG pathway could have a role in these. In migrating fibroblasts, CG endosomes are
453 localized to the leading edge and transient ablation of these endosomes inhibits
454 efficient migration²⁶. Increase in the membrane tension at the leading edge keeps the
455 neutrophil cells polarized and helps in its migration⁵⁴. In a separate study in
456 neutrophils, GBF1 localizes to the leading edge by binding to products of
457 phosphatidylinositol 3-kinase (PI3K), recruits ARF1, and this localization is needed
458 for unified cell polarity⁵⁵. We have found that PI3K products help recruit GBF1 to the
459 plasma membrane and this is necessary for CG endocytosis⁵⁶. Thus, one could
460 speculate that a polarized CG pathway and its regulators operating in migrating cells
461 could be modulating membrane tension by regulating membrane trafficking.

462 Endocytic pathways are proposed to be at the core of a eukaryotic cellular plan
463 integrating multiple inputs over spatio-temporal scales⁵⁷. They are also necessary for
464 tissue patterning: the CG pathway is utilized for Wingless signaling for patterning of
465 the *Drosophila* wing disc during larval development⁵⁶. Here we find that a high
466 capacity CG pathway that turns over a huge fraction of the plasma membrane^{26,32} and
467 sensitive to membrane composition³² is modulated by temperature and
468 mechanochemical inputs. A vinculin-mediated negative feedback loop between
469 membrane tension and the CG pathway helps maintain the cell at a lower tension set
470 point (Cartoon: Fig 8b). This could also help in increasing the potential for
471 modulating membrane tension to regulate other cellular processes. Thus, the CG
472 pathway responds and coordinates a variety of cellular inputs including membrane
473 tension and is likely to function in multiple physiological contexts.

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500 AUTHOR CONTRIBUTIONS

501 J.J.T and S.M conceived the study. J.J.T, A.J.K, P.P, P.R-C, M.A.d.P, R.G.P and S.M
502 designed the experiments. J.J.T, A.J.K, A.E-A and N.C performed the experiments
503 and analyzed them. M.C.G and R.G.P performed the EM experiments designed by
504 R.G.P and M.A.d.P. S.P and P.P built the optical tweezer setup. X.T and P.R-C
505 designed and built the stretch system. S.S, P.P.S and R.V synthesized LG186. J.J.T
506 and S.M wrote the paper.

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660 **FIGURE LEGENDS**

661 **Figure 1: A fast transient endocytic response to decrease in membrane tension:**

662 (a) Cartoon showing membrane remodeling responses after mechanical strain. Cells
663 after the stretch and relax protocol forms invaginations termed ‘reservoirs’⁶. These
664 reservoirs are resorbed in few minutes by an active process and requires ATP. (b) The
665 illustration shows the longitudinal section of a vacuum based equi-bi-axial stretching
666 device. Cells plated on a PDMS sheet are stretched by the application of controlled
667 vacuum below the circular PDMS sheet, which stretches it in a calibrated manner.
668 Releasing the vacuum relaxes the strain on PDMS thus relaxing the cell. Cells plated
669 on PDMS can be imaged in an upright or inverted microscope as required. (c)
670 Endocytic response on strain relaxation. CHO cells were pulsed for 90 sec with TMR-
671 Dex at steady state (steady state), immediately on relaxing the stretch (stretch-relax),
672 or after a waiting time of 90 seconds after relaxing the stretch (stretch-relax-wait).
673 After the pulse, cells were quickly washed with ice cold buffer and fixed, followed by
674 imaging on a wide field microscope. Images show representative cells used to
675 generate the histograms which provide a quantitative measure of the extent of
676 endocytosis of TMR-Dex for the indicated treatments (d) Endocytic response on
677 deadhering. CHO cells are pulsed with TMR-Dex for 3 minutes in adhered cells
678 (Spread), during de-adhering (Deadh), or immediately after cells are detached and in
679 suspension (Suspension) were washed with ice cold buffer, added back to
680 concanavalin coated glass bottom dishes in ice cold buffer, fixed and imaged on a
681 wide field microscope. Images and Histogram show the extent of fluid-phase uptake
682 under the indicated conditions. In each experiment, the data represent the mean
683 intensity per cell (\pm S.D) from two different experiments with duplicates containing at
684 least 100 cells per experiment. *: $P < 0.001$, ns: not significant. Scale bar, 10 μ m.

685 **Figure 2: Endocytic pathways differ in their response to decrease in membrane
686 tension:**

687 (a) Fluid-phase and transferrin (Tf) uptake in CHO cells on recovery from osmotic
688 shock. Fluid-phase and Transferrin uptake were monitored in CHO cells under
689 Isotonic conditions (Iso) or immediately after shifting from the hypotonic to isotonic
690 state (Hypo-Iso) by incubating cells with A647-Tf (Transferrin) or TMR-Dex (Fluid)
691 as indicated in Methods. Wide-field images (left) show the extent of endocytosed
692 fluid-phase in Isotonic or Hypotonic-Isotonic (Hypo-Iso) conditions. Histogram
693 (right) show the extent of TMR-Dex and A647-Tf endocytosis in the Hypo-Iso
694 condition normalized to those measured in the isotonic condition (grey dashed line).
695 (b) Fluid-phase and Tf uptake in CHO cells on deadhering. CHO cells were pulsed
696 with TMR-Dex (Fluid) and A647-Tf (Transferrin) for 3 minutes when the cells are
697 adherent (Spread) or during detachment (Deadh), and taken for imaging as described
698 in Methods. Wide-field images (left) show the extent of endocytosed fluid-phase in
699 Spread and during de-adhering condition (Deadh). Histogram (right) show the extent
700 of TMR-Dex and A647-Tf endocytosis in the de-adhered condition normalized to that
701 measured in the Spread condition (grey dashed line). (c) GPI-AP and Tf uptake on
702 deadhering in CHO cells. CHO cells pulsed with fluorescent folate to label cell
703 surface GPI-anchored folate receptors (GPI-AP) and with A647-Tf (Transferrin) for 3
704 minutes at 37 °C in normally adherent cells (Spread) or during detachment (Deadh),
705 and taken for imaging as described in Methods. Wide-field images (left) show the
706 extent of endocytosed GPI-anchored folate receptor in Spread and during the

707 deadhhering condition. Histogram (right) shows the extent of A647-Tf and Folate
708 receptor endocytosis in the de-adhered condition normalized to those measured in the
709 Spread condition (grey dashed line). In each experiment, the data represent the mean
710 intensity per cell (\pm S.D) from two different experiments with duplicates containing at
711 least 100 cells per experiment. *: $P < 0.001$, ns: not significant. Scale bar, 10 μ m.

712 **Figure 3: CLIC/GEEC (CG) pathway is the primary pathway for fast endocytic
713 response:**

714 **(a)** Endocytic response in WT, dynamin TKO, and caveolin null MEFs on stretch-
715 relax. Wild type MEF (WT MEF), Caveolin^{-/-} (Cav^{-/-}), or conditional Dynamin triple
716 knock out cells (Dyn TKO) were pulsed for 90 sec with TMR-Dex at steady state,
717 immediately after relaxing the stretch (stretch-relax), and quickly washed with ice
718 cold buffer, fixed and imaged on a wide field microscope. Images (left) show
719 representative cells used to generate the histograms (right) which provide a
720 quantitative measure of the extent of endocytosis of TMR-Dex for the indicated
721 treatments. The uptake on stretch-relax in each cell line is plotted normalized to the
722 steady state uptake in the respective cell lines (grey dashed line). **(b)** Inhibition of CG
723 pathway and endocytosis on stretch-relax. CHO cells were treated with DMSO
724 (Control) or with LG186 (10 μ g/ml) to inhibit GBF1 for 30 minutes prior to pulsing
725 with TMR-Dex for 90 sec, either at steady state (steady state), or immediately after
726 relaxing the stretch (stretch-relax), and quickly washed with ice cold buffer, fixed and
727 imaged on a wide field microscope. Images show representative cells used to generate
728 the histograms (right) which provide a quantitative measure of the extent of
729 endocytosis of TMR-Dex for the indicated treatments, normalized to the control
730 steady state condition (grey dashed line). In each experiment, the data represent the
731 mean intensity per cell (\pm S.D) from two different experiments with duplicates
732 containing at least 100 cells per experiment. *: $P < 0.001$, ns: not significant. Scale
733 bar, 10 μ m.

734 **Figure 4: Temperature dependence of CG pathway and reservoir resorption:**

735 **(a)** Endocytosis of fluid-phase and Tf with temperature. CHO cells pre-equilibrated
736 at the indicated temperatures were pulsed with TMR-Dex (Fluid) and Tf-A647
737 (Transferrin) for 5 minutes at the respective temperatures, and the extent of
738 endocytosis of the two probes were quantified and normalized to those obtained at 37
739 °C (grey dashed line). Representative images (left) of cells used to generate the
740 histogram (right) ($\text{mean} \pm \text{S.D}$) were obtained from two different experiments with
741 duplicates each containing at least 100 cells per experiment. Scale bar, 10 μ m. **(b)**
742 Reservoir resorption on inhibiting CG pathway and decreasing temperature. The
743 reservoir fluorescence intensity after stretch relax of CHO cells transfected with a
744 fluorescent membrane marker (pEYFP-mem) was quantified as a function of time at
745 37°C in the absence (37°C control) or presence of LG186 (37°C inhibitor), or at room
746 temperature (26°C control). Each point represents mean \pm S.E.M from more than 100
747 reservoirs from at least 10 cells. Scale bar, 10 μ m.

748 **Figure 5: CG pathway regulates membrane tension:**

749 **(a)** Cartoon shows a membrane tether attached to a polystyrene bead trapped in an
750 optical trap, used to measure tether forces. The polystyrene bead is held using a laser
751 based optical trap to pull membrane tethers from cells. Displacement of the bead from

752 the center of the trap (Δx) gives an estimate of the tether force (F) of the cell using the
753 Hook's law ($F = -k * \Delta x$; where k is the spring constant or trap stiffness; see methods).
754 Membrane tension is obtained from the steady state force as $\sigma = F^2/(8\pi^2 B)$, where B
755 is the bending modulus of the membrane and F is the tether force⁵⁸. **(b)** Tether forces
756 on downregulating CG pathway by GBF1 inhibition. Membrane tethers were pulled
757 from CHO cells pre-treated with DMSO (CHO Control) or LG186 (CHO LG186) for
758 30 minutes, and maintained at 37 °C during the measurement. Tether forces were
759 calculated as indicated above. The box plot shows data points with each point
760 corresponding to a tether per cell with data combined (n = 16 (CHO control) and 19
761 (CHO LG186)) from two different experiments. **(c, d)** Endocytosis (c) and tether
762 forces (d) in dynamin TKO cells. Wild type (WT) MEF, or conditional Dynamin
763 TKO cells were either pre-treated with DMSO or LG186 and were pulsed for 5
764 minutes with TMR-Dex and taken for imaging (c) or directly taken to measure tether
765 forces (d). The histograms shows fluid-phase uptake normalized to that observed in
766 untreated WT MEF cells (c) and box plot shows tether forces (d) measured in the
767 indicated conditions (n = 25 (WT MEF), 19 (DYN TKO) and 22 DYN TKO LG186)).
768 **(e)** Endocytosis on modulating CG pathway. CHO cells were treated without BFA
769 (Control), or with BFA (20 μ g/ml) alone or with LG186 for 30 minutes, and then
770 directly incubated with TMR-Dex for 5 minutes, and imaged on a wide field
771 microscope. The histogram shows fluid-phase uptake per cell normalized to control
772 treated CHO cells (Dashed grey line). HeLa cells were correspondingly treated with
773 BFA and the histogram shows the extent of fluid-phase uptake under the indicated
774 conditions, normalized to that observed in untreated HeLa cells. **(f)** Box plot shows
775 tether forces measured in CHO or HeLa cells treated without (Control) or with BFA
776 for 45 minutes (n = 17 (CHO Control), 23 (CHO BFA), 18 (HeLa Control), 19 (HeLa
777 BFA)). **(c, e)** In each endocytosis experiment, the data represent the mean intensity
778 per cell (\pm S.D) from two different experiments with duplicates containing at least
779 100 cells per experiment. *: $P < 0.001$, ns: not significant.

780 **Figure 6: Mechanical modulation of CG molecular machinery**

781 **(a)** WT- MEF cells transfected with GBF1-GFP were imaged live by TIRF
782 microscopy. They exhibit GBF1-punctae at the plasma membrane, which is
783 modulated by alterations in osmolarity obtained by changing the media from isotonic
784 (Iso) to 40% hypotonic (Hypo) and back to isotonic (Iso). **(b)** Quantification of the
785 number of punctae per cell during hypotonic shock and subsequent shift to isotonic
786 medium. The GBF1 spots upon hypotonic shock and subsequent shift to isotonic
787 medium is normalized to number of spots in the respective cell determined before
788 hypotonic shift and plotted as a box plot. Each data point is a measurement from a
789 single cell and box plot shows data of 12 cells from two independent experiments.
790 Scale bar, 10 μ m.

791 **Figure 7: Vinculin dependent mechanoregulation of CG pathway**

792 **(a)** Vinculin null cells were pulsed for 90 seconds with F-Dex either during a 6%
793 stretch or on relaxing this strain. Fluid-phase uptake per cell during this strain change
794 is normalized to uptake in steady state cells for the same time point, and plotted in the
795 left panel. Representative images are shown in the right panel. **(b)** WT MEF (left
796 panel) and vinculin null cells (right panel) are pulsed with F-Dex for 2 minutes in
797 increasing hypotonic medium as indicated, washed, fixed and imaged. Average
798 uptake per cell in the indicated hypotonic media was normalized to the average uptake

799 of the isotonic condition and plotted as a box plot. Note while fluid-phase uptake is
800 inhibited in WT MEF cells proportional to the increase in hypotonicity, vinculin null
801 cells are refractory to much higher hypotonicity. **(c)** WT or vinculin null MEFs are
802 pulsed with F-Dex for 3 minutes as described before at 37°C. Left panel shows
803 quantification of uptake per cell normalized to the WT levels and right panel shows
804 the representative images of the same. In each endocytosis experiment, the data
805 represent the mean intensity per cell (\pm S.D) from 2 independent experiments
806 with each with two technical duplicates containing at least 100 cell per
807 experiment. *: $P < 0.001$, ns: not significant. Scale bar, 10 μ m. **(d)** Vinculin null
808 cells transfected with GBF1-GFP and imaged live using TIRF microscopy while
809 changing media from isotonic (Iso) to 40% hypotonic (Hypo) and back to isotonic
810 (Iso). GBF1 organization at plasma membrane during the osmotic shifts is shown in a
811 representative cell (left panel). Quantification of the number of punctae per cell
812 during hypotonic and isotonic shifts is normalized to number of spots (Grey dotted
813 line) before hypotonic shift of the respective cells is plotted as a box plot (right
814 panel). Each data point is measurement from a single cell and box plot shows data of
815 13 cells from two independent experiments. Scale bar, 10 μ m.

816 **Figure 8: Membrane tension and Vinculin**

817 **(a)** WT (Vin $+/+$) or vinculin null cells (Vin $-/-$) were treated with LG186 to inhibit
818 GBF1 mediated CG pathway and membrane tension measured using optical tweezer
819 as described before and compared to the control treated cells. ($n = 20$ (Vin $+/+$), 25
820 (Vin $+/+$ with LG186), 25 (Vin $-/-$) and 29 (Vin $-/-$ with LG186)). Vinculin null cells
821 shows a higher basal membrane tension compared to WT MEF; inhibiting the CG
822 pathway drastically reduced membrane tension in both cell lines. **(b)** CG pathway and
823 membrane tension operates in a vinculin dependent negative feedback loop to
824 maintain homeostasis. Reduction of tension from its steady state leads to a passive
825 response by the formation of reservoirs or VLDs. The decrease in the effective
826 tension inactivates a vinculin-dependent machinery, resulting in an increase in active
827 GBF1, which increases the CG pathway and rapid internalization of the excess
828 membrane. This is a fast transient response that appears to restore the steady state. On
829 the other hand, increasing the membrane tension from steady state activates vinculin
830 dependent machinery, inhibiting the CG pathway, via the reduction of GBF1
831 recruitment. The increase in effective tension could also activate exocytic machinery
832 which adds membrane resulting in restoration of the steady state. Thus, a vinculin
833 dependent mechanochemical-regulation of the CG pathway through a negative
834 feedback loop helps in maintaining plasma membrane tension homeostasis.
835

836 **SUPPLEMENTARY FIGURE LEGENDS**

837 **Supplementary Figure 1:** (a) Fluid-phase endocytosis is inhibited on stretching of
838 cells. WT – MEF cells grown on the PDMS device as detailed in Methods, were
839 subjected to 6% strain and pulsed for 90 seconds with TMR-Dextran (TMR-Dex)
840 under the stretched condition (stretch), fixed and imaged. Fluorescence intensity is
841 compared to the cells that were not subjected to the strain (control) cells pulsed for 90
842 seconds. (b) Recycling of the fluid-phase is not affected during deadheling. Cartoon
843 describes the pulse chase protocol used to look at recycling during deadheling. CHO
844 cells are pulsed with TMR-Dex for 2 minutes, washed and chased for 5 minutes either
845 in their adhered steady state (Spread) or during de-adhering (Deadh) were washed,
846 fixed, and imaged. Histogram (right) shows TMR-Dex remaining in the cells after
847 chase in the deadheling or in spread condition normalized to the spread condition. (c)
848 Shifting from hypotonic to isotonic media results in an increase in fluid-phase uptake.
849 Cells were pulsed with TMR-Dex for 1 minute either in steady state (Iso) or after
850 hypotonic shock (hypo-iso) for one minute. Images (left) of endocytosed TMR-Dex
851 and histogram (right) show the extent of fluid-phase uptake. In each experiment, the
852 data represent the mean intensity per cell (\pm S.D) from two different experiments with
853 duplicates containing at least 100 cells per experiment. *: $P < 0.001$, ns: not
854 significant. Scale bar, 10 μ m.

855 **Supplementary Figure 2:** (a) Dynamin TKO cells as detailed in Methods (Dyn
856 TKO), or (Control) was pulsed with Tf-A568 for 5 minute, surface stripped, labelled
857 with antibody against transferrin receptor (TfR), washed and fixed. Transferrin (Tf)
858 uptake normalized to the TfR levels is plotted in the histogram (right top) along with
859 the surface TfR normalized to the control (right bottom), determined per cell.
860 Corresponding wide field images show extent of endocytosed TfR (top) and surface
861 TfR (bottom). Note that removal of all Dynamin isoforms inhibits transferrin uptake
862 comprehensively while increasing surface levels of TfR. (b) Dynamin TKO cells (as
863 in panel a) were pulsed with TMR-Dex for 1 minute either in steady state (control) or
864 after hypotonic shock (hypo-iso). Wide-field images (left) show extent of TMR-Dex
865 uptake and the histogram (right) shows fluid-phase uptake per cell in hypo-iso
866 condition normalized to the control. Note while uptake of TfR is completely inhibited
867 (a), fluid-phase uptake (b) exhibits a typical increase on shifting from hypotonic to
868 isotonic conditions in Dynamin TKO cells. (c) MEFs (Cav-/-) were pulsed with TMR-
869 Dex for 3 minutes when they are normally adherent cells (Spread) or during
870 detachment (Deadh). Wide field images and histogram show the extent of fluid-phase
871 uptake under these conditions. (d) Electron micrographs of WT-MEF and Cav1-/-
872 MEF on deadheling show similar CG endosomes. CTxB-HRP uptake for 5 minutes
873 and processed for DAB reaction as described in methods is done in both wild type
874 MEF (WT-MEF) and Cav1-/- MEF. Arrows show internalized CG carriers in both
875 cell types and arrow head show surface connected caveolae in de-adhered WT cells
876 (inset shows zoomed in image).

877 In each experiment, the data represent mean intensity per cell (\pm S.D) from two
878 different experiments each with technical duplicates containing at least 100 cells per
879 experiment. *: $P < 0.001$. Scale bar, 10 μ m (a, b, c), 500nm (d).

880 **Supplementary Figure 3:** (a) ML141, a small molecule inhibitor of CDC42, inhibits
881 fluid-phase uptake but not transferrin uptake at steady state. CHO cells treated
882 without (Control) or with ML141 (10 μ M) for 45 minutes were pulsed for 5 minutes

883 with TMR-Dex (Fluid) and A488-Tf (Tf), washed and surface-stripped of remnant-
884 cell surface Tf. These cells were incubated with A647-labelled OKT9 antibody on ice
885 to detect surface transferrin receptor (TfR Surface). Wide field images of cells (left)
886 and the histogram of total fluid-phase and Tf uptake normalized to surface receptor
887 level shows that the effect of ML141 was only on the fluid-phase uptake but not on
888 TfR endocytosis. **(b)** Inhibiting CG pathway using ML141 prevents increase in fluid-
889 phase uptake on deadhering. Adherent CHO cells treated without (Control) or with
890 ML141 (10 μ M) for 45 minutes were pulsed for 5 minutes with TMR-Dex without
891 detachment (Spread) or during the deadhering process (Deadh), and washed
892 extensively, fixed and taken for imaging. Images (left) of endocytosed TMR-Dex and
893 histogram (right) show the extent of fluid-phase uptake normalized to that observed in
894 the control spread cells. Note that increase in endocytosis observed while deadhering
895 is completely abolished upon inhibition of CG endocytosis by ML141. **(c)** LG186, a
896 small molecule inhibitor of GBF1, inhibits fluid-phase uptake but not transferrin
897 uptake. CHO cells treated without (Control) or with LG186 (10 μ M) for 30 minutes
898 were pulsed for 5 minutes with TMR-Dex (Fluid) and A488-Tf (Tf), washed
899 extensively and surface-stripped of remnant-cell surface Tf. These cells were
900 incubated with A647-labelled OKT9 antibody to detect surface transferrin receptor
901 (TfR Surface). Wide field images of cells (left) and histogram of total fluid-phase
902 uptake and Tf uptake normalized to surface receptor level shows that the effect of
903 LG186 was only on the fluid-phase uptake but not on TfR endocytosis. **(d)** Inhibiting
904 CG pathway using LG186 prevents increase in fluid-phase uptake on deadhering.
905 Adherent CHO cells treated without (Control) or with LG186 (10 μ M) for 30 minutes
906 were pulsed for 5 minutes with TMR-Dex without detachment (Spread) or during the
907 deadhering process (Deadh), and washed, fixed and taken for imaging. Images (left)
908 of endocytosed TMR-Dex and histogram show the extent of fluid-phase normalised to
909 that observed in the control cells. Note that increase in endocytosis observed while
910 deadhering is completely abolished upon inhibition of CG endocytosis by inhibiting
911 GBF1 using LG186. In each experiment, the data represent the mean intensity per cell
912 (\pm S.D) from two different experiments with duplicates containing at least 100 cells
913 per experiment. **(e)** CD44 uptake is inhibited at high tension. WT MEFs were pulsed
914 CD44 mAb for 2 minutes either in isotonic medium (Iso) or in 75mOsm hypo-
915 osmotic medium (Hypo) at 37°C, acid washed to strip surface mAb, washed and
916 labeled with AF-555 secondary antibody. (Histogram (right) shows per cell uptake in
917 hypo-osmotic situation normalized to the isotonic situation for the representative
918 images shown on left. Data represents mean \pm S.E.M from three independent
919 experiments).*: $P < 0.001$, ns: not significant. Scale bar, 10 μ m.

920 **Supplementary Figure 4:** **(a)** Fluid-phase uptake in HeLa cells is insensitive to
921 GBF1 inhibition by LG186 treatment. HeLa cells treated without (Control) or with
922 LG186 (10 μ M) for 30 minutes were pulsed for 5 minutes with TMR-Dex (Fluid) and
923 washed extensively, or pulsed with A488-Tf (Tf) and surface-stripped of remnant-cell
924 surface Tf, followed by labeling with A648-labelled OKT9 antibody to detect surface
925 transferrin receptor (TfR Surface). Wide field images of cells (left) and histogram of
926 total fluid-phase uptake and Tf uptake normalized to surface receptor level shows that
927 LG186 does not have an effect on the fluid-phase uptake and TfR endocytosis in
928 HeLa cells. Data represent the mean intensity per cell (\pm S.D) from two different
929 experiments with duplicates containing at least 100 cells per experiment. Scale bar, 10
930 μ m. **(b/c)** GBF1 do not efficiently localize to the plasma membrane nor form punctae.
931 GBF1-GFP was transfected in either CHO or HeLa cells and imaged in TIRF and in

932 wide field (epi-fluorescence) format. HeLa cells do not show surface punctae
933 formation unlike CHO cells (b). Quantification of the TIRF to wide field ratio of
934 GBF1-GFP levels shows (c) that GBF1 translocation to surface is much lower in
935 HeLa cells. Each point is value from a cell and quantified from two different
936 experiments (n = 23 (CHO) and 20 (HeLa)). (d) Rapid shifting from hypotonic to
937 isotonic state does not result in an increase in fluid-phase uptake in HeLa Cells. HeLa
938 cells were pulsed with TMR-Dextran (fluid) for 1 minute either in steady state (Iso) or
939 after hypotonic shock of one minute (Hypo-Iso). Images (left) of endocytosed TMR-
940 Dex and histogram show the extent of fluid-phase uptake. Data represent the mean
941 intensity per cell (\pm S.D) from two different experiments with duplicates containing at
942 least 100 cells per experiment. (e) Reservoir adsorption in HeLa cells does not
943 respond to GBF1 inhibition. The reservoir fluorescence intensity after stretch relax of
944 HeLa cells transfected with a fluorescent membrane marker (pEYFP-mem) was
945 quantified as a function of time at 37°C in the absence (37°C control) or presence of
946 LG186 (37°C inhibitor), or at room temperature (26°C control). Each point represents
947 mean \pm s.e.m from more than 200 reservoirs from at least 10 cells. Note while
948 treatment with LG186 had no effect on the rate of reservoir resorption, lowering of
949 the temperature also had only a marginal reduction in the rate of resorption. Scale bar,
950 10 μ m.

951 **Supplementary Figure 5:** (a) Reservoirs formed on stretch-relax does not colocalize
952 with endosomes. A pH sensitive SecGFP-GPI transfected CHO cells are stretched for
953 one minute and relaxed in pH 7.4 buffer (pH 7, top left) at 37°C showing formation of
954 reservoirs as before. The pH is made acidic (pH 5.5) after 30 seconds to quench
955 surface fluorescence. Only internal vesicles at neutral pH remain fluorescent and thus
956 helps to capture newly formed endosomes on stretch-relax (pH 5, top right). The
957 reservoirs does not colocalize with the newly formed endosomes (bottom, insets). (b)
958 The cells transfected with CAAX-GFP to mark the membrane are plated on glass
959 (left) or polyacrylamide gels. Cells were incubated with hypo-osmotic medium for
960 one minute followed by isotonic recovery. Images of VLDs are observed only in the
961 cells grown on glass. Insets show a magnification of the edge of the cells where VLDs
962 are expected to form. (c) Endocytic pulse of 90 seconds is done either in isotonic
963 situation or after release of hypotonic shock of 1 minute in cells plated on glass or gel.
964 Mean uptake per cell after release of hypotonic shock for either gel or glass is
965 normalized to its uptake in isotonic conditions (dotted line). Data represent the mean
966 intensity per cell (\pm S.D) from two different experiments with duplicates containing at
967 least 100 cells per experiment. (d) Cartoon depicts a magnetic tweezer set up to
968 measure membrane stiffness. An electromagnet with a sharpened tip was used to
969 apply a 0.5 nN pulsatory force (1 Hz) to paramagnetic beads coated with either
970 fibronectin fragment (FN) or ConcanavalinA (ConA), attached to cells as described in
971 methods. Bead movement in response to applied force was then tracked, and bead
972 stiffness was calculated during the first 10 seconds of the measurement in units of
973 nN/ μ m. (e, f) Inhibiting of CG pathway with LG186 lowers membrane stiffness.
974 ConA (e) or Fibronectin fragment (f) coated paramagnetic beads were attached to
975 cells treated without (Control) or with LG186 for 30 minute. Beads were pulled using
976 the magnetic tweezer as described, and membrane stiffness calculated. Note LG186
977 treatment lowers membrane stiffness only when ConA-coated beads are pulled,
978 whereas FN-coated beads are unaffected. This indicates that the LG186 treatment
979 does not disrupt the cortical cytoskeleton that interacts with the integrin-engaged FN-
980 coated beads, but lowers general membrane tension as experienced by the ConA-

981 coated beads. The box plot shows the stiffness values in nN/ μ m in each of the tethers
982 pulled for the different conditions. (g) BrefeldinA (BFA) treatment causes disruption
983 in GM130 distribution in both HeLa cells and CHO cells. Cells were treated with 20
984 μ g/ml of BFA for 45 minute, fixed, permeabilized and labelled with GM130 antibody
985 to mark cis-Golgi and imaged on a wide field microscope. BFA treatment disrupts the
986 peri-nuclear localization in both CHO and HeLa cells, indicating that BFA is
987 functional in both cell types. (h) The increase in fluid-phase uptake in Dynamin TKO
988 is inhibited by LG186 treatment. WT or Dynamin TKO cells (prepared as described
989 in methods) were treated with LG186 or with the vehicle (DMSO; Control), and
990 incubated with TMR-Dex for 5 minutes, washed and fixed prior to imaging on a Wide
991 Field microscope. The images show that while Dyn TKO cells exhibit a higher fluid-
992 phase uptake than the WT cells, this uptake is sensitive to the inhibition of GBF1,
993 confirming that it is the CG endocytosis that has a higher activity in Dynamin TKO
994 cells (quantified in Fig. 5c). Scale bar, 10 μ m.

995 **Supplementary Figure 6:** (a) WT cells transfected with clathrin light chain (CLC-
996 mCherry) was imaged live using TIRF microscopy during osmotic changes as
997 indicated. Images (Left panel) and graph (right) show the quantification of number of
998 CLC punctae per cell normalized to the steady state number of punctae from 13 cells.
999 Note that the number of clathrin pits at the cell surface do not respond to changes in
1000 tension. Scale bar, 10 μ m. (b) GBF1 punctae in vinculin null cells are higher. GBF1-
1001 GFP is transfected in either WT-MEF or vinculin null MEF and imaged in TIRF. The
1002 number of punctae per cell were counted and normalized to the surface area. The plot
1003 shows the average number of spots in each cell type normalized to those obtained in
1004 WT-MEF. Each point in the box plot represents measurements collected from a single
1005 cell (WT MEF, n= 12; (VIN NULL, n= 13), accumulated. (c) Cells were pulsed with
1006 F-Dex either during deadhering or in spread state for 3 minutes and the pulse is
1007 stopped using ice cold buffer as described before. De-adhered cells are added to vial
1008 containing ice cold buffer and allowed to attach on a fresh coverslip bottom dish to be
1009 fixed and imaged. Endosomal intensity per cell is quantified and normalized to the
1010 intensity of the WT spread. Note that Vinculin null cells show higher endocytosis
1011 compared to WT cells but does not show increase in uptake on deadhering unlike WT
1012 cells. (d) Vinculin null cells rescued by transfection with WT-Vinculin full length
1013 (VIN+) and vinculin null cells were treated with LG186 to check sensitivity to GBF1
1014 inhibition of fluid-phase endocytosis. Cells treated with LG186 for 30 minutes were
1015 pulsed for 5 minutes with F-Dex, washed, fixed and imaged. Endocytic uptake per
1016 cell is quantified and after normalizing to the untreated Vin null case (Control) is
1017 plotted as shown. Note adding back vinculin reduces the fluid-phase uptake. However
1018 in both in the Vinculin null as well as in the cells in which Vinculin is added back,
1019 endocytic uptake of the fluid-phase remains sensitive to LG186, indicating a role for
1020 vinculin in negatively regulating the CG pathway. Data represent the mean intensity
1021 per cell (\pm S.D) from two different experiments with technical duplicates with at least
1022 100 cells per experiment. *: $P < 0.001$, ns: not significant.

1023 **Supplementary Figure 7:** (a) Vinculin cells were either untreated (Control) or
1024 treated with either LG186 (LG186), BFA (BFA) or BFA followed by LG186
1025 treatment (BFA LG186). Cells were pulsed with F-Dex for 3 minutes and uptake per
1026 cell quantified as detailed in Methods, normalized to control, and plotted in the
1027 histogram (left). The histogram and Images (right) show that BFA treatment causes an
1028 increase in fluid-phase uptake in vinculin null cells and treating with LG186 inhibits

1029 fluid-phase uptake even in BFA treated cells. This indicates that the CG pathway is
1030 operational and capable of being upregulated in the vinculin null cells. Data represent
1031 the mean intensity per cell (\pm S.D) from two different experiments with duplicates
1032 containing at least 100 cells per experiment. *: $P < 0.001$, ns: not significant. Scale
1033 bar, 10 μ m. **(b)** Model for maintaining membrane tension homeostasis. Different
1034 processes such as endocytosis, hypotonic shock, and adhesion increase effective
1035 membrane tension (arrow headed line) while exocytosis, hypertonic medium and
1036 deadhering would reduce the effective membrane tension (bar headed line). This
1037 effective membrane tension is a physical parameter that activates a vinculin
1038 dependent mechano-transduction machinery. Depending on the change in tension in
1039 relation to a steady state set point, this mechanotransduction machinery inhibits the
1040 activity of G_BF1 at the cell surface (red bar headed line). Higher effective membrane
1041 tension in relation to the steady state would activate vinculin mediated machinery and
1042 inhibit G_BF1. G_BF1 levels at the cell surface is crucial for the activity of the CG
1043 pathway and thus directly regulates the level of the endocytosis (green arrow headed
1044 line). Thus any modulation of the membrane tension could be balanced by this
1045 negative feedback loop to maintain tension homoeostasis.
1046

1047 **METHODS**

1048 **Cell Culture and reagents**

1049 The CHO (Chinese Hamster Ovary) cells stably expressing FR-GPI and human
1050 transferrin receptor TfR (IA2.2 cells) as described before³³, HeLa, MEF (Mouse
1051 embryonic fibroblasts) cells, Caveolin null MEF, conditional null dynamin triple
1052 knockout MEF, vinculin null MEF were used for the assays. HF12 media (HiMEDIA,
1053 Mumbai, India) and DMEM (Invitrogen) supplemented with NaHCO₃ and L-
1054 Glutamin/Penicillin/Streptomycin solution (Sigma Aldrich) was used for growing
1055 CHO cells and the different MEF lines respectively.

1056 BrefeldinA (BFA) (Sigma Aldrich), ML141 (Tocris Bioscience) and LG186 (see
1057 synthesis section below) dissolved in DMSO was used at 20 µg/ml, 10µM and 10µM
1058 respectively. ML141 and LG186 treatment was done for 30 minutes in serum free
1059 media and maintained during endocytic assays. Tetra methyl rhodamine labelled
1060 dextran (TMR-Dex) (10,000MW;Molecular probes, ThermoFisher Scientific) was
1061 used at 1mg/ml. 4 – hydroxy tamoxifen (Sigma Aldrich) was used at 3 µM to remove
1062 Dynamin 1/2/3 from the conditional dynamin triple knockout MEF cells as reported
1063 previously ²⁹. TrypLE express (GIBCO, Invitrogen) was used to detach cells
1064 according to manufacturer's instruction. SYLGARD 184 silicone elastomer kit (Dow
1065 Corning) was used to make PDMS sheets according to manufacturer's instruction. For
1066 the reservoir experiments, cells were transfected with a membrane targeting plasmid
1067 pEYFP-mem (Clontech) using the Neon transfection device according to the
1068 manufacturer's protocol as described earlier ⁶.

1069 **Endocytic and Recycling assays on deadhering**

1070 Endocytic assays were done as previously described^{28,33} with slight modifications as
1071 required. Briefly, CG endocytosis was monitored using fluorescent dextran at 1mg/ml
1072 in medium or fluorescent folate analogue (N^α-pteroyl-N^ε-Bodipy^{TMR}-L-lysine
1073 (PLB^{TMR})) in folate free medium for indicated time points at 37°C. Endocytosis of
1074 TfR was monitored using 10 µg/ml fluorescent transferrin (Tf) at 37°C incubation for
1075 indicated time points. Endocytosis was stopped using ice cold HEPES based
1076 buffer(M1) (M1:140mM NaCl, 20mM HEPES, 1mM CaCl₂, 1mM MgCl₂, 5mM KCl,
1077 pH 7.4). To remove surface fluorescence, cells were treated with PI-PLC (50µg/ml,
1078 1h; GPI-APs) or with ascorbate buffer (160mM sodium ascorbate, 40mM ascorbic
1079 acid, 1mM MgCl₂, 1mM CaCl₂, pH 4.5; Tf) at 4°C and subsequently fixed with 4%
1080 paraformaldehyde for 10 minutes.

1081 To study endocytosis on deadhering, cells were detached using TrypLE containing
1082 fluorescent dextran at 1mg/ml concentration for 3 minutes and the detached cells were
1083 pipetted into an ice cold vial containing M1 buffer to stop the endocytosis. Cells were
1084 then replated back on coverslip bottom dish maintained at 4°C, fixed, washed and
1085 imaged. To look at endocytosis in suspension, the cells soon after detaching were
1086 pipetted into vial containing fluorescent dextran kept at 37°C. The volume was
1087 adjusted to have a final concentration of 1mg/ml of the TMR-Dex and after 3minutes
1088 the endocytosis was stopped by shifting vial to ice. The cells are spun down at 4°C
1089 and then re-plated on coverslip bottom dish coated with ConA, maintained at 4°C,
1090 fixed, washed and imaged.

1091 To understand recycling of cargo on deadhering, cells were pulsed with F-Dex for 3
1092 minutes, quickly washed with M1 buffer at room temperature and then detached with
1093 TrypLE at 37°C for 5minutes, pipetted into vial containing ice cold M1 buffer and
1094 kept on ice. Cells were then re-plated back on coverslip bottom dish coated with
1095 ConA maintained at 4°C, fixed, washed and imaged.

1096 For different small molecule inhibitors used, the cells were treated with them for 30
1097 minutes in serum free media in their respective final concentrations and then medium
1098 was removed and pulsed with F-Dex at 1mg/ml in serum free media containing the
1099 inhibitors since the inhibitor activity is reversible. Endocytosis is stopped by washing
1100 with ice cold M1 buffer, fixed and imaged.

1101 **CTxB-HRP uptake, DAB reaction and Electron Microscopy**

1102 WT and Cav^{-/-} MEFs were de-adhered at room temperature followed by
1103 internalization of 4 $\mu\text{g.ml}^{-1}$ CTxB-HRP (Invitrogen) at 37°C for 5 minutes, washed
1104 two times with ice cold PBS followed by incubation on ice for 10 minutes with
1105 1mg.ml⁻¹ DAB(Sigma-Aldrich) with 50 μM Ascorbic acid. This is followed by a 10
1106 minute treatment with DAB, Ascorbic acid and 0.012% H₂O₂ and then washed twice
1107 with ice cold PBS. Cells were fixed using 2.5% Glutaraldehyde (ProSciTech) at room
1108 temperature (RT) for 1 hour followed by PBS wash for two times and then washed
1109 with 0.1M Na cacodylate and left in the same for overnight at 4°C. Cells were
1110 contrasted with 1% osmium tetroxide and 4% uranyl acetate. Cells were dehydrated in
1111 successive washes of 70%, 90% and 100% ethanol before embedding using 100%
1112 LX-112 resin at 60°C overnight. Sections were viewed under a transmission electron
1113 microscope (JEOL 1011; JEOL Ltd. Tokyo, Japan), and electron micrographs were
1114 captured with a digital camera (Morada; Olympus) using AnalySIS software
1115 (Olympus).

1116 **Preparation of PDMS membrane ring**

1117 Sylgard 184 silicone elastomer kit comes in two parts which are added in 10 to 1 mix
1118 ratio between the polydimethylsiloxane base and the curing agent. This is thoroughly
1119 mixed and degassing is done either using a vacuum desiccator or by centrifugation.
1120 To prepare PDMS sheets, 7ml of this mixture was added to the middle of a circular
1121 6inch plate which is spun at 500R.P.M for a minute on a spin coater. This was cured
1122 at 65°C overnight and then carefully peeled off either after treatment with oxygen
1123 plasma cleaner for 40 seconds or without treatment. This PDMS sheet is spread
1124 evenly and tightly placed between rings of the stretcher (Fig 1a). The cells were
1125 plated in the middle of the PDMS sheet surrounded with water soaked tissue paper to
1126 retain humidity and prevent drying up of medium. These rings were placed in the
1127 stretcher and stretched by varying the level of vacuum as needed according to the
1128 calibration for the experiments.

1129 **Stretch and osmolarity experiments**

1130 For the stretch relax experiments, cells plated on PDMS membrane were loaded on
1131 the cell stretcher system (Fig 1a) within a temperature controlled chamber at 37°C.
1132 Vacuum was applied beneath the ring containing the PDMS sheet, deforming the
1133 membrane and stretching the cells plated on the PDMS. The setup was calibrated to
1134 stretch cells equi-biaxially to cause 6% strain. Medium containing F-Dex was kept at

1135 37°C in a water bath, and used for the endocytic pulse for the indicated time during or
1136 after stretch (see endocytic protocol above). Control cells were treated in the same
1137 way except for application of stretch.

1138 For the osmolarity experiments, cells were treated with 50% hypotonic medium made
1139 with deionized water at 37°C for the indicated time points and then pulsed with F-Dex
1140 either in hypotonic or isotonic medium as needed. The shock is applied for 60 seconds
1141 and pulse done for 60 seconds as mentioned. Endocytosis was stopped with ice cold
1142 M1 buffer, washed, fixed and imaged.

1143 **Optical Tweezer measurements**

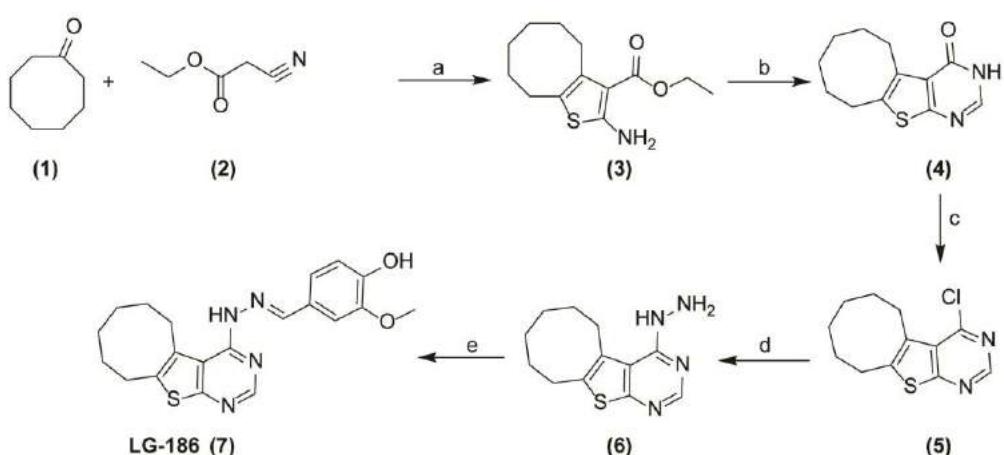
1144 Tether forces was measured using an custom built optical tweezer using IR laser
1145 (CW,1064nm, TEM_00,1W) along with 100x, 1.3NA oil objective and motorized
1146 stage on a Olympus IX71 inverted microscope. Polystyrene beads added to the
1147 imaging chamber were allowed to settle and then held in the optical trap while
1148 simultaneously imaging through bright field on a coolsnap HQ CCD camera.
1149 Membrane tethers are formed by attaching the beads to the cells for few seconds and
1150 by moving the bead away using the piezo stage. The tether is held at a constant length
1151 and the fluctuation in the trapped bead is detected by using a quadrant photodiode
1152 which in turn is acquired and saved using a Labview program through a Data
1153 Acquisition Card (USB-6009 NI). The trap stiffness is calibrated using the power
1154 spectrum method. The displacement of the bead from the center along with the trap
1155 stiffness is used to calculate the tether forces live using a custom written Labview
1156 code.

1157 **Magnetic Tweezer measurements**

1158 Magnetic tweezer measurements were carried out as previously described⁵⁹. Briefly, a
1159 custom-built electromagnet with a sharpened tip was used to apply a 0.5 nN pulsatory
1160 force (1 Hz) to paramagnetic beads attached to cells. Paramagnetic beads had been
1161 previously coated with either the fibronectin fragment FN7-10 or concanavalin A
1162 (Sigma Aldrich), which bind respectively specifically to integrins and the actin
1163 cytoskeleton or nonspecifically to the membrane through glycoproteins⁶⁰. Bead
1164 movement in response to applied force was then tracked, and bead stiffness was
1165 calculated during the first 10 seconds of the measurement as the transfer function
1166 between the applied force and the resulting displacement, evaluated at 1 Hz. This
1167 stiffness thus provides a measure of the resistance to force of the cell-bead adhesion
1168 in units of nN/μm.

1169 **Synthesis of LG186**

1170 LG186 was synthesized freshly prior to experiments as reported previously ³⁶ with
1171 slight modification as described below and used at 10μM dissolved in DMSO..



1172
1173 **Reagent and conditions:** Reagents and compounds obtained are mentioned as
1174 numbers and the conditions for the reaction are labelled alphabetically along with
1175 arrows in figure. a) S_8 , morpholine, ethanol reflux 6 h; b) $HCONH_2$, 150°C, 5 h; c)
1176 $POCl_3$, DMF, rt. d) Hydrazine hydrate, methanol, rt. 2 h; e) Vanillin, rt. 2 h.
1177

1178 **Compound 3:** To a solution of cyclooctanone (1) (10 mmol) in ethanol (10 mL)
1179 were added sulfur (10 mmol), ethyl cyanoacetate (2) (10 mmol) and morpholine (4
1180 mmol). The reaction mixture was stirred at 60°C for 5 h. Upon completion of reaction
1181 (checked by TLC), evaporate the solvent and extracted with ethyl acetate and purified
1182 by column chromatography using dichloromethane. 1H NMR (400 MHz, $CDCl_3$) δ :
1183 1.28 (m, 5H), 1.39 (m, 2H), 1.50 (m, 2H), 1.56 (m, 2H), 2.54 (m, 2H), 2.75 (m, 2H),
1184 4.21 (q, 2H), 5.86 (brs, 2H). LC-MS: 254 ($M+H$)⁺.
1185

1186 **Compound 4:** Compound 3 was heated at 150°C in 5 mL formamide for 5 h. Upon
1187 cooling overnight, the product crystallized as slightly brownish crystals. The resulting
1188 crystals were collected and washed with a mixture of cold ethanol/water (1/1) to give
1189 the corresponding product in quantitative yield. 1H NMR (400MHz, DMSO) δ :
1190 1.27(m, 2H), 1.42 (m, 2H), 1.62(m, 4H), 2.87 (m, 2H), 3.06 (m, 2H), 8.5 (s, 1H), 11.4
1191 (brs, 1H). LC-MS: 234.9 ($M+H$)⁺.
1192

1193 **Compound 5:** Compound 4 was dissolved in hot DMF and then ice-cooled prior to
1194 the addition of $POCl_3$ (2 equivalents). Upon stirring overnight, the product
1195 precipitated out as white solid 5, which was collected and washed with cold water and
1196 used for next step without purification.
1197

1198 **Compound 7:** Compound 5 was dissolved in methanol and then added 10 equivalent
1199 of hydrazine monohydrate. The mixture was stirred for 2 h and water was added. The
1200 resulting precipitate was filtered off and washed with cold water to obtain compound
1201 6, which was then treated with 1.2 equivalent of vanillin. The mixture was stirred for
1202 2 h, diluted with water and extracted with dichloromethane. The organic layer was
1203 dried with $MgSO_4$, filtered off and concentrated in vacuo and purified by column
1204 chromatography using ethyl acetate: hexane (4:6).
1205 1H NMR (400MHz, DMSO) δ : 1.27 (m, 2H), 1.46 (m, 2H), 1.62 (m, 2H), 1.68 (m,
1206 2H), 2.85 (m, 2H), 3.19 (m, 2H), 3.88 (s, 3H), 6.84 (d, 1H), 7.60 (d, 1H), 7.79 (s, 1H),
8.30 (s, 1H), 9.45 (brs, 1H) 11.70 (brs, 1H). ^{13}C NMR: ^{13}C NMR (100 MHz, DMSO) δ

1207 149.29, 148.97, 148.47, 146.76, 146.41, 141.11, 135.01, 132.59, 122.39, 118.77,
1208 117.05, 116.46, 113.05, 58.20, 56.24, 26.95, 25.84, 25.72, 25.50. LC-MS: 383
1209 (M+H)⁺.

1210 **Imaging, Analysis and Statistics**

1211 The quantification of endocytic uptake for a population is done by imaging on 20x,
1212 0.75 NA on a Nikon TE300 wide field inverted microscope. For the stretch
1213 experiments, an upright microscope (Nikon eclipse Ni -U) was used with a water
1214 immersion objective (60x, 1.0 NA). Different fields were obtained and cells within
1215 the field were outlined as regions using either metamorph or micromanager software.
1216 The images were analyzed using MetaMorph® or Micro-Manager software and is
1217 processed for presentation using Adobe Illustrator. All images displayed are equally
1218 scaled for intensity unless otherwise mentioned. The scale bar is 10 μ m unless
1219 otherwise mentioned. The integrated intensities, spread area and thus average uptake
1220 per cell were determined using the region measurement option. Each value plotted
1221 here is mean value from two different experiments with duplicates in each and
1222 standard deviation between the two experiments. Statistical significance was tested
1223 using Mann-Whitney test and p-values are reported.

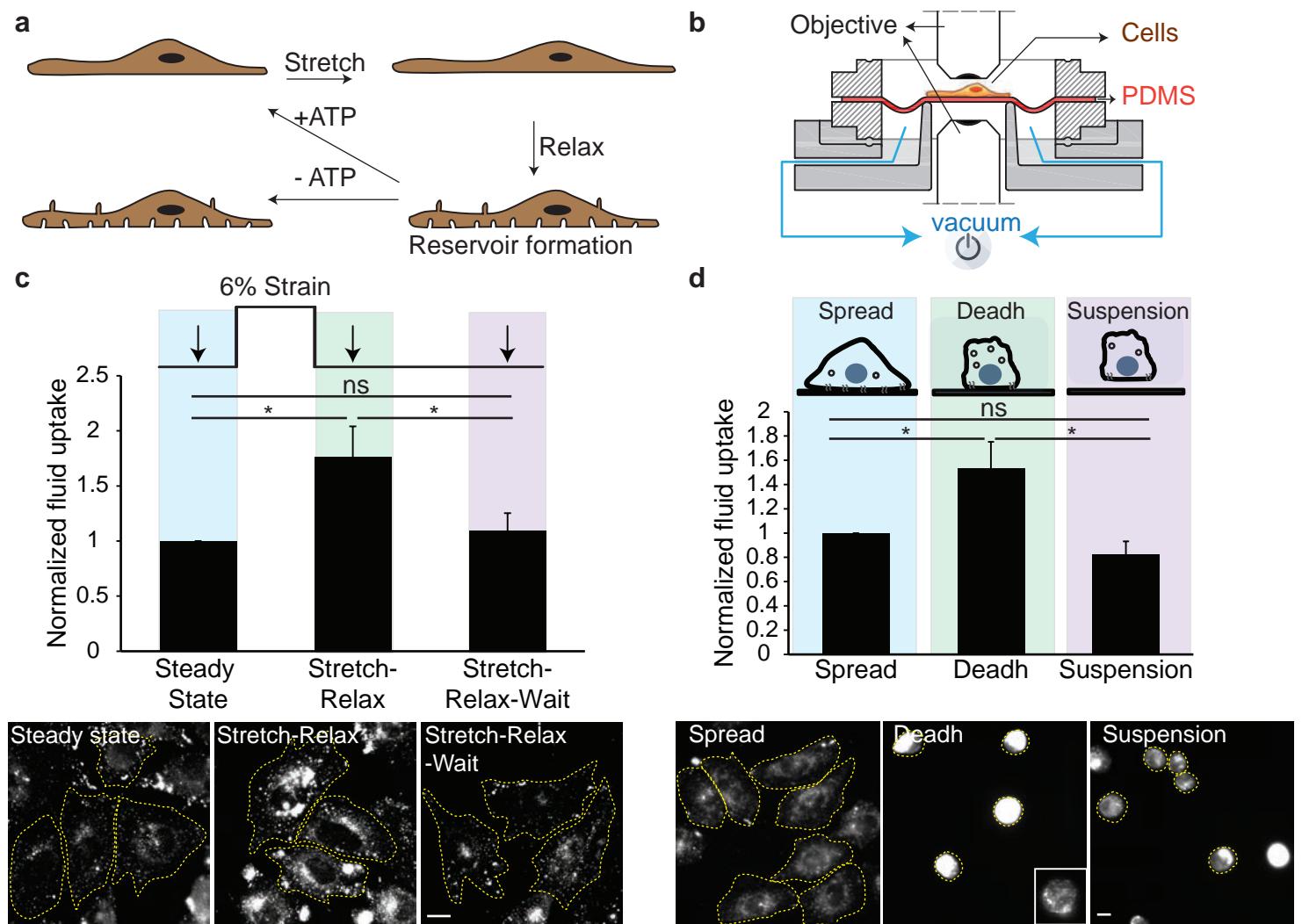


FIGURE 1

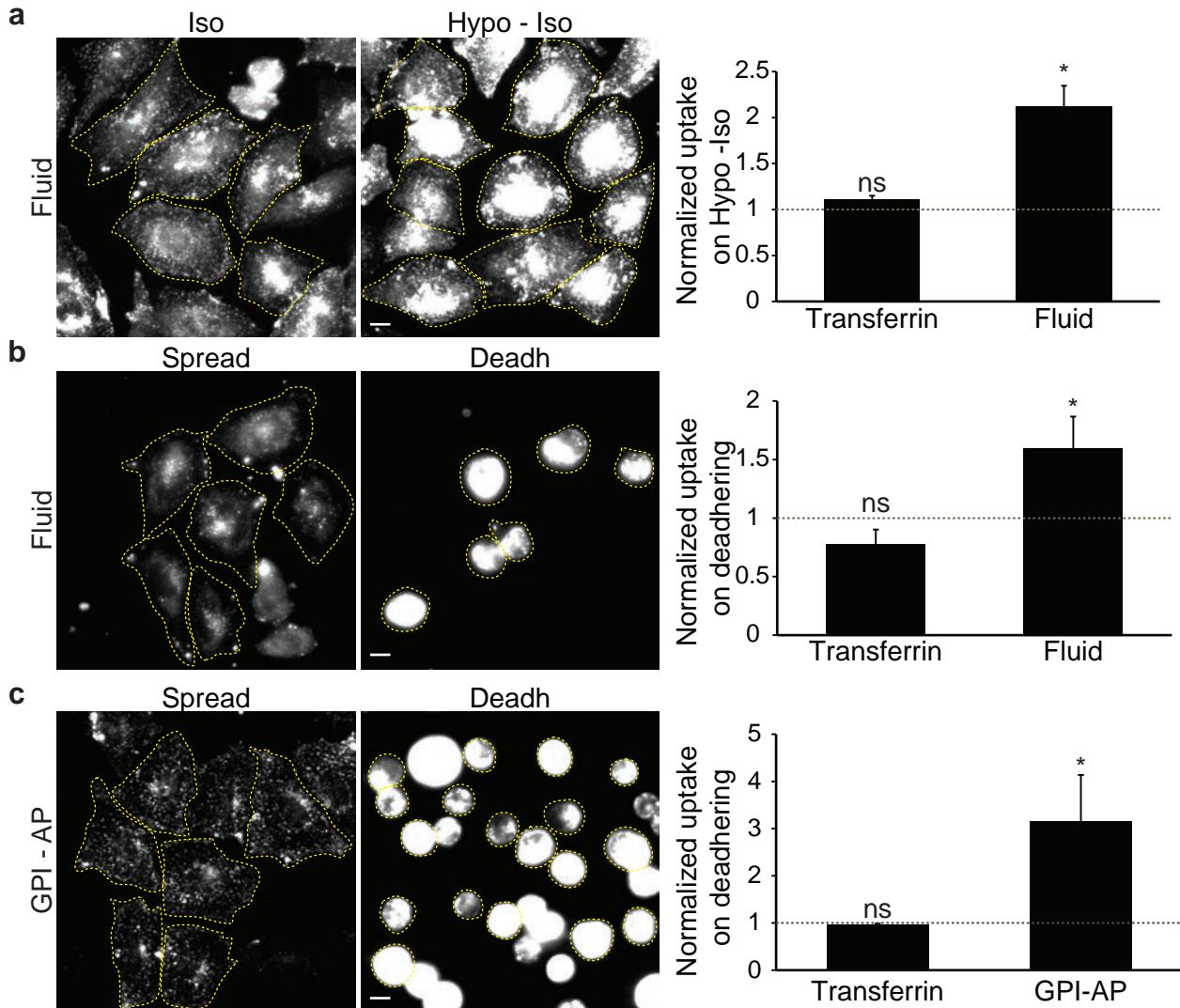


FIGURE 2

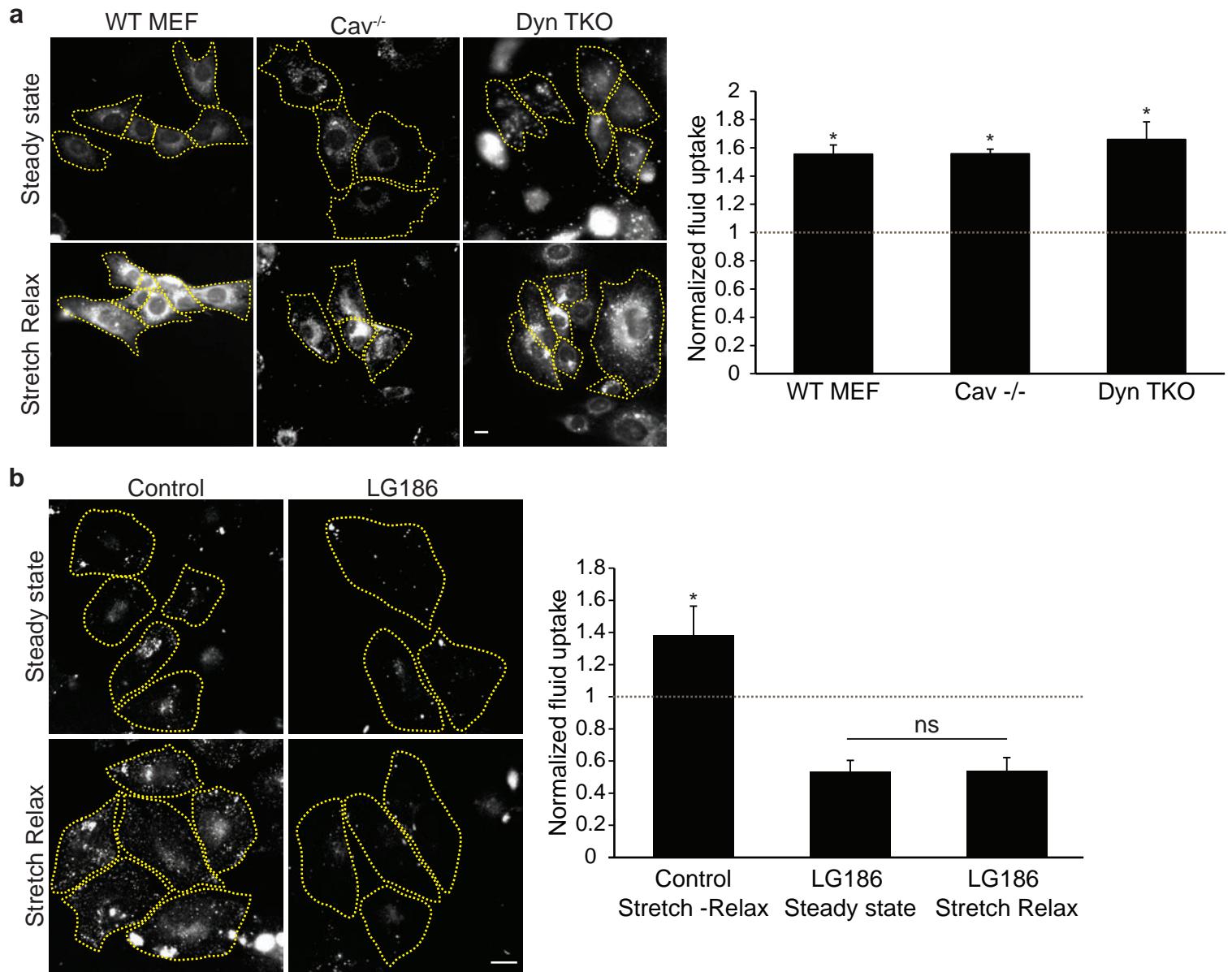


FIGURE 3

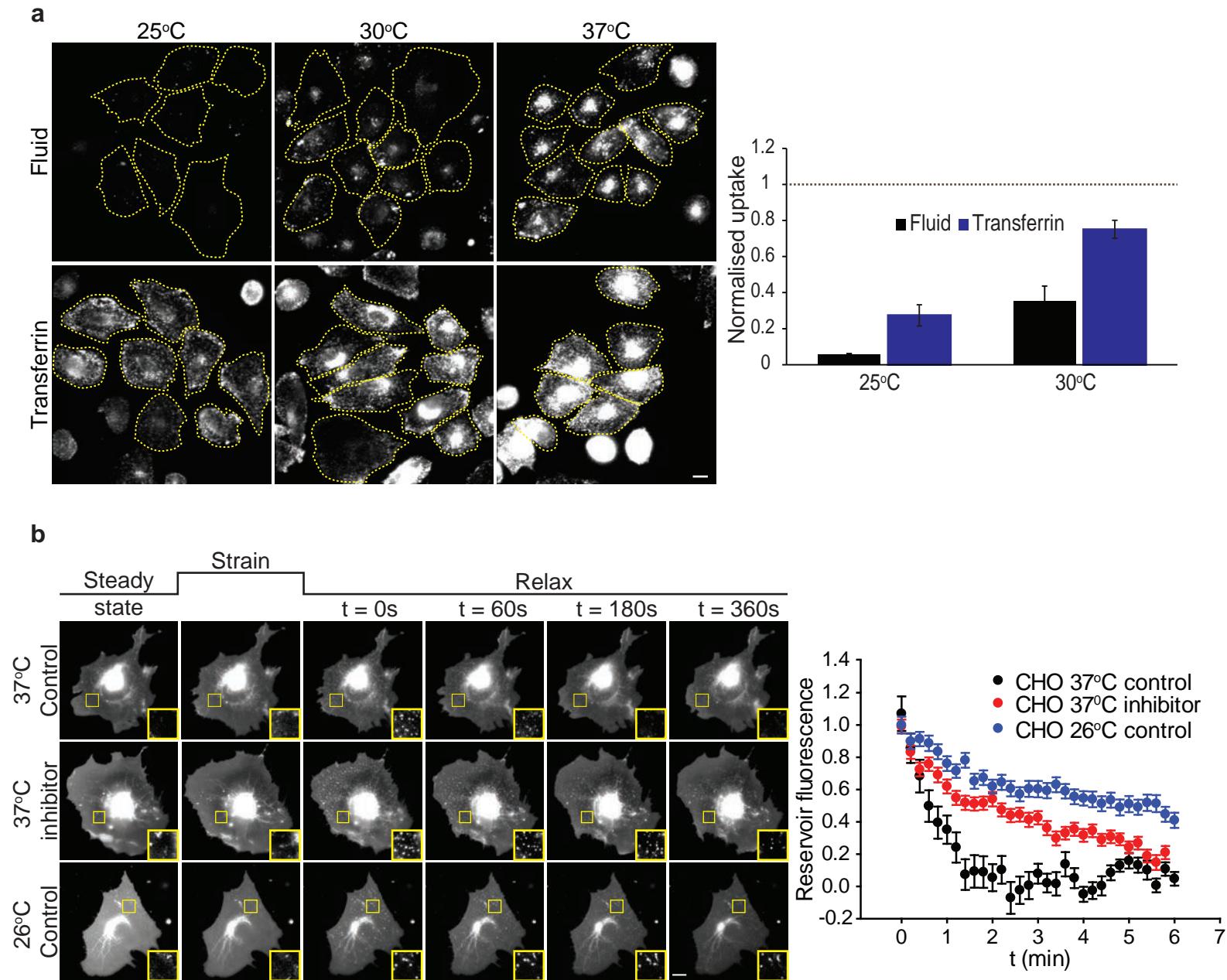


FIGURE 4

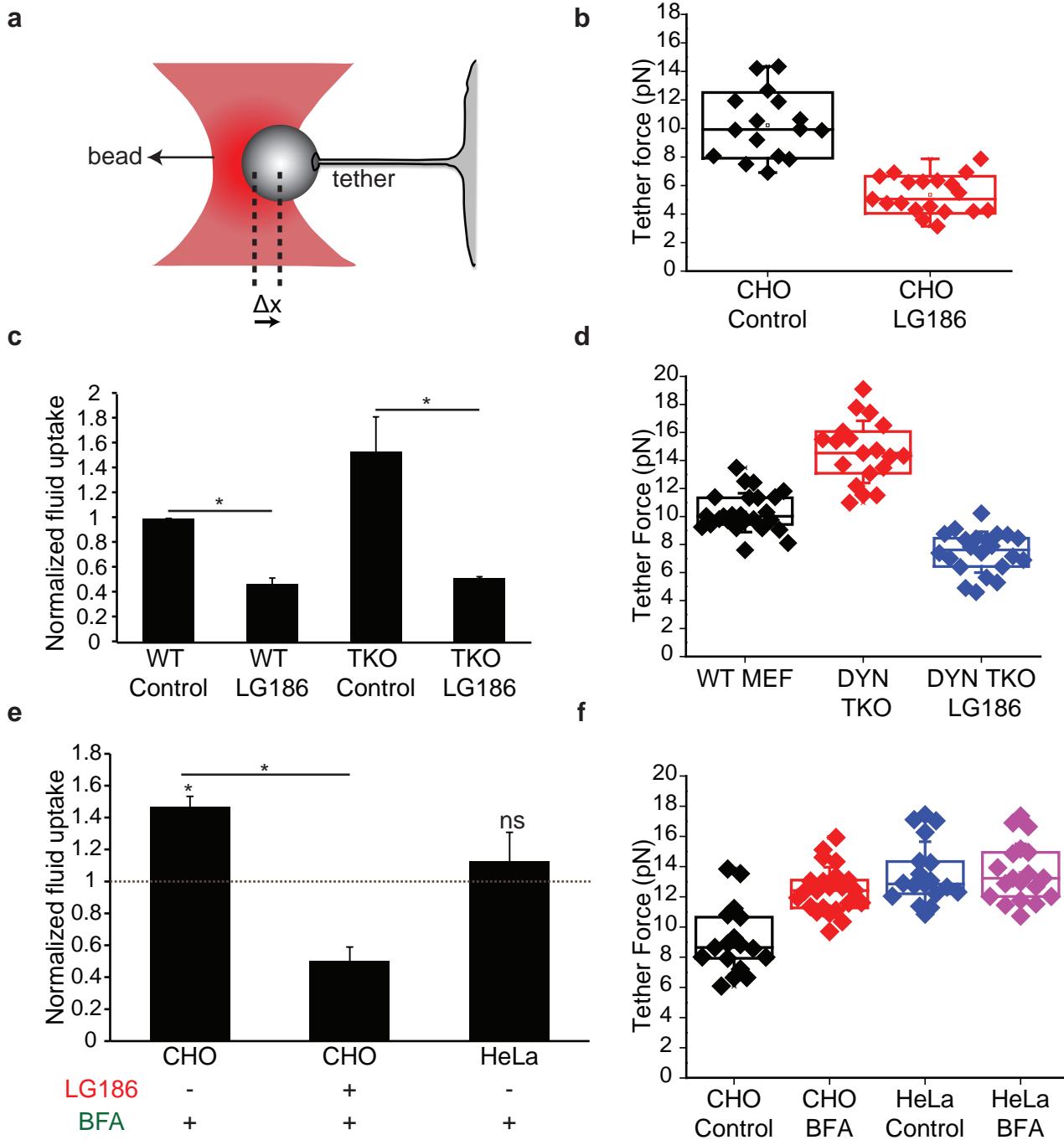


FIGURE 5

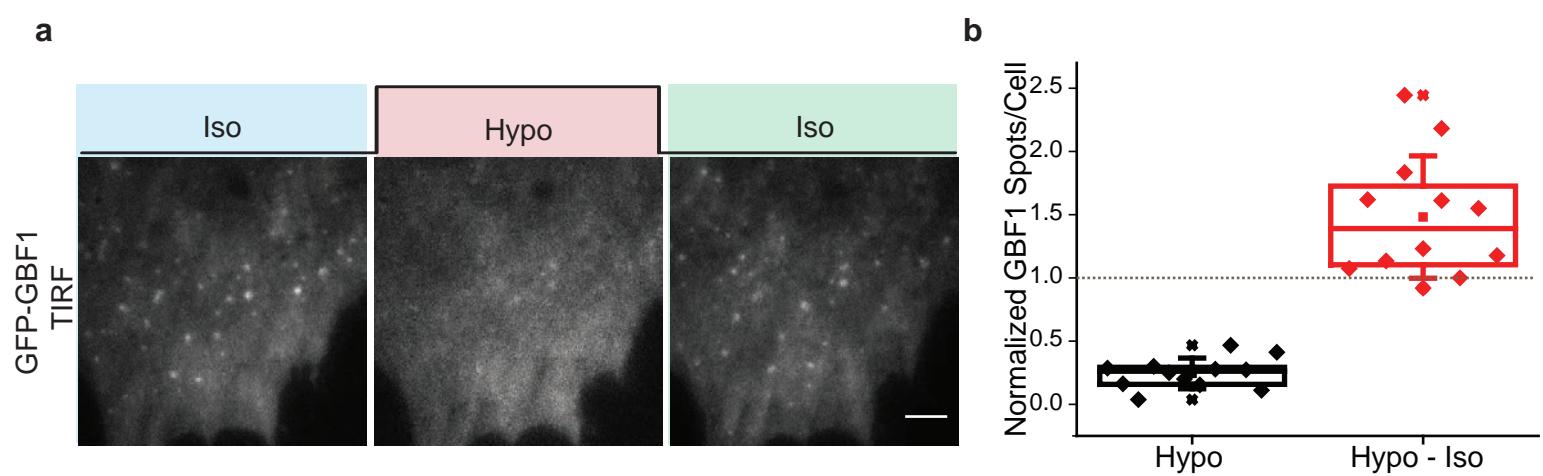


FIGURE 6

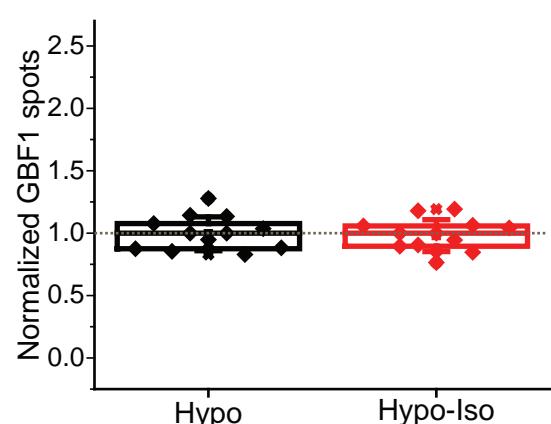
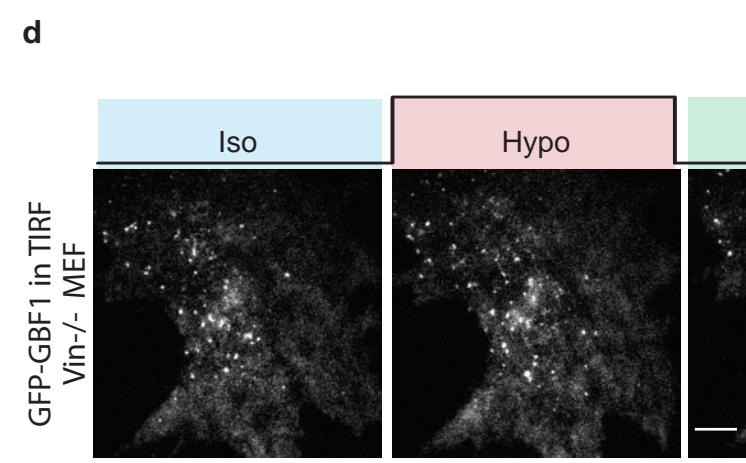
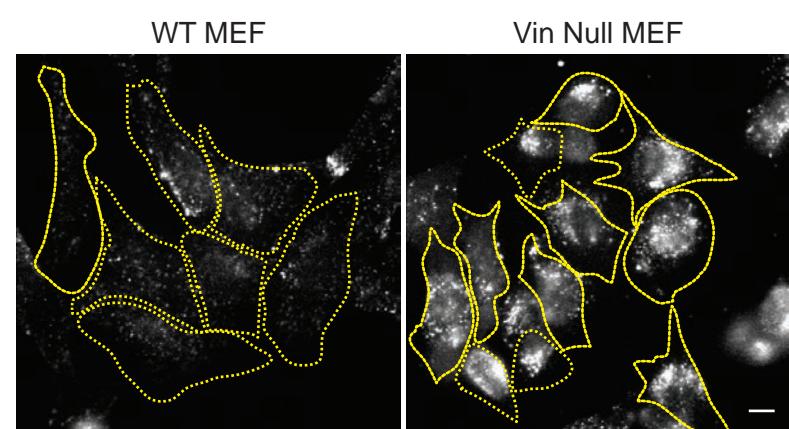
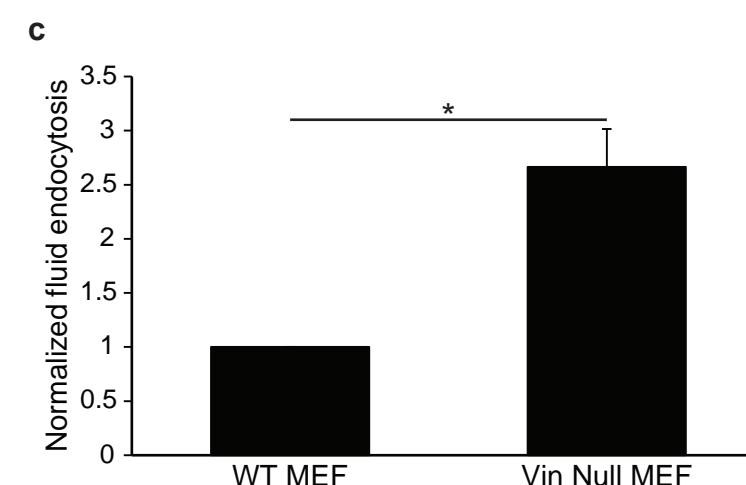
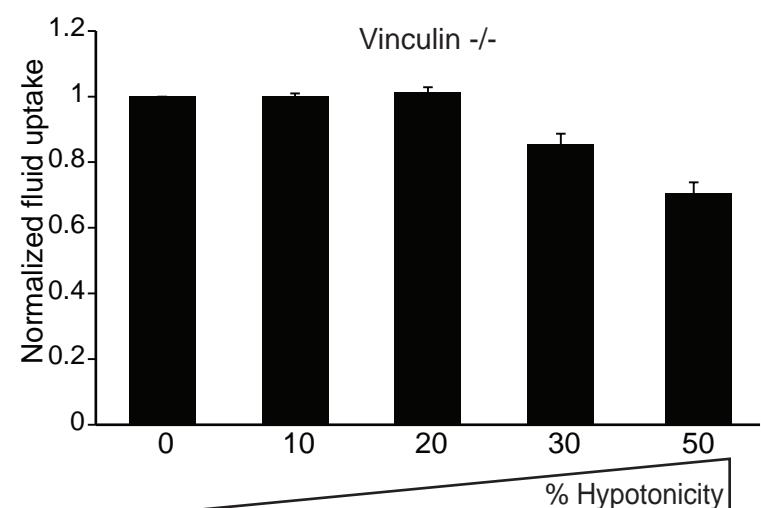
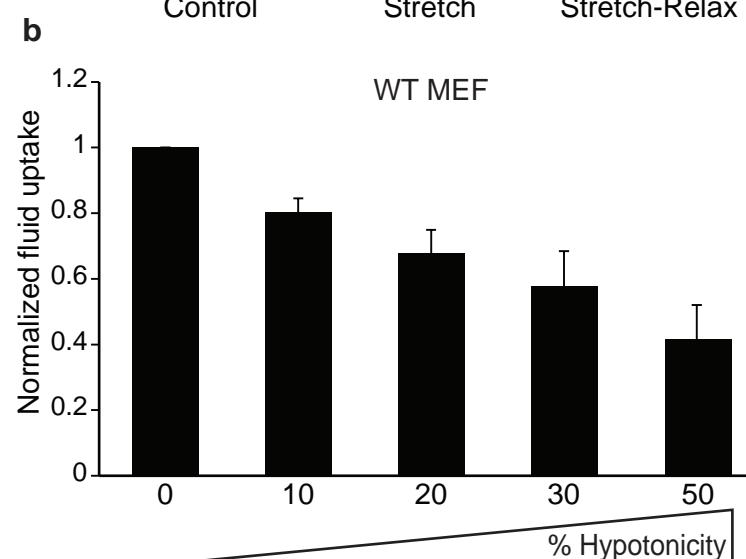
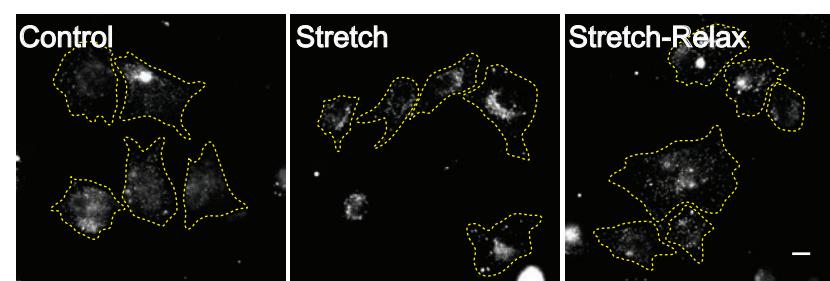
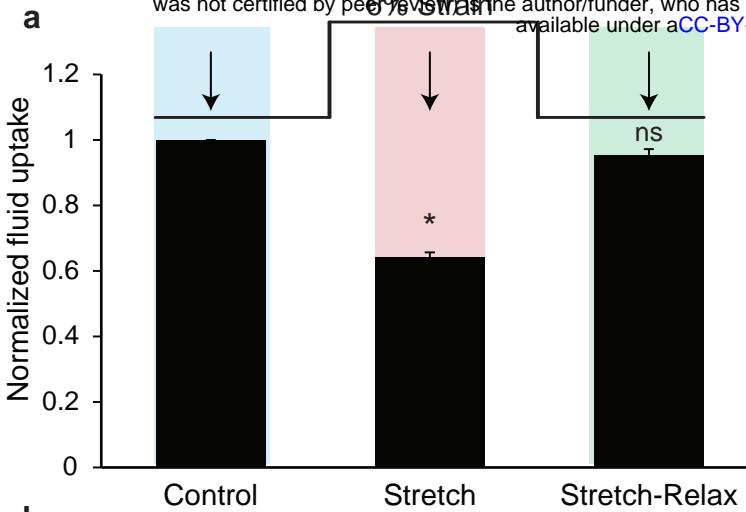
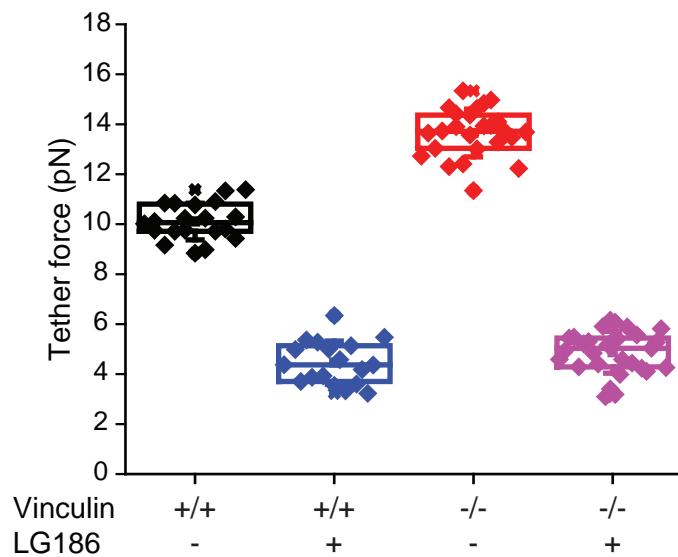


FIGURE 7

a



b

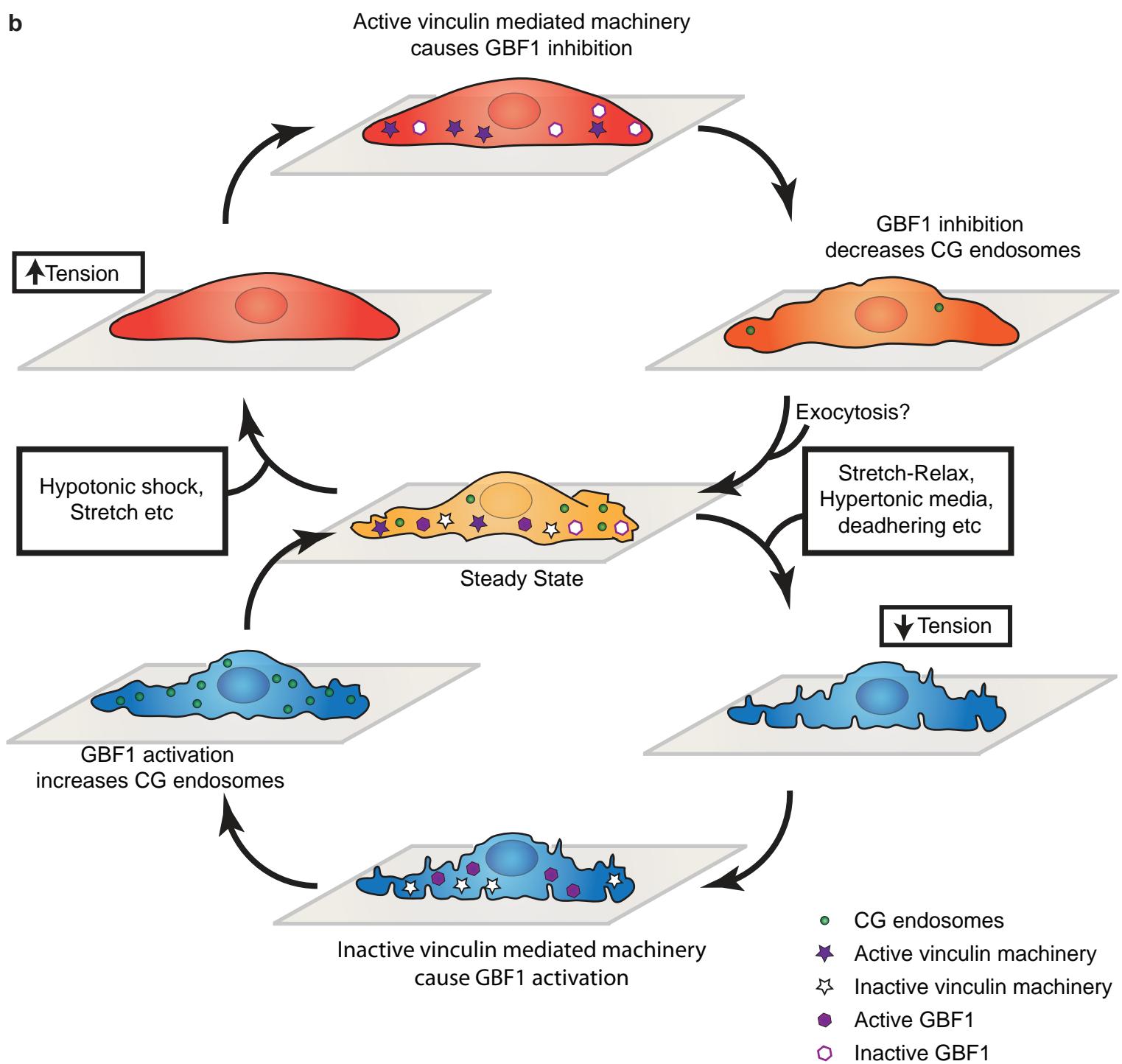
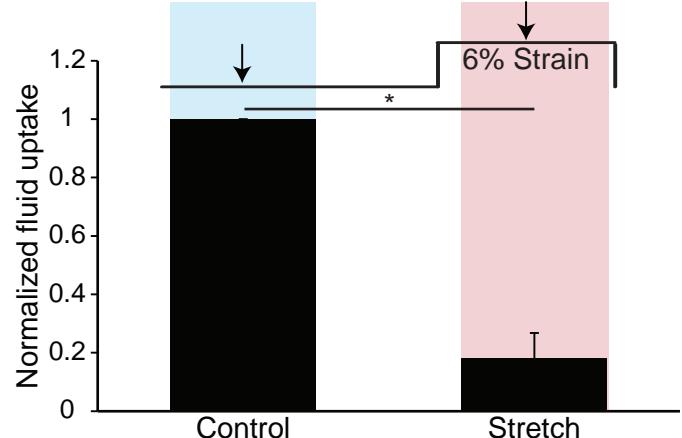
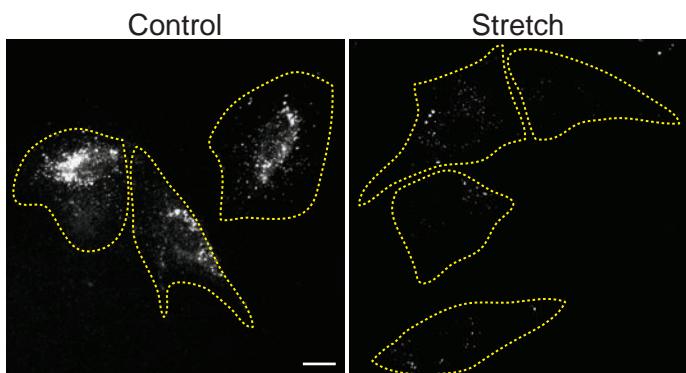
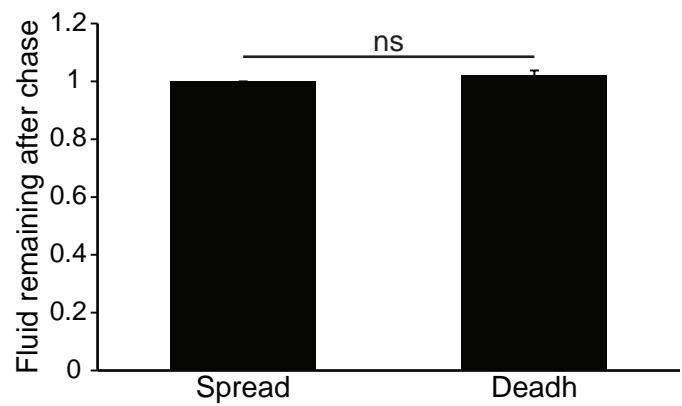
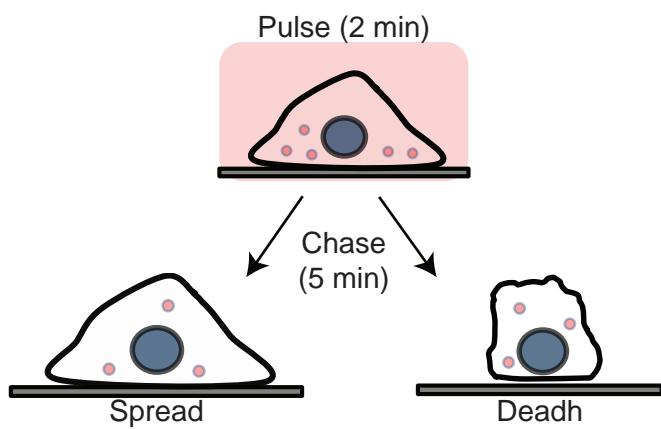


FIGURE 8

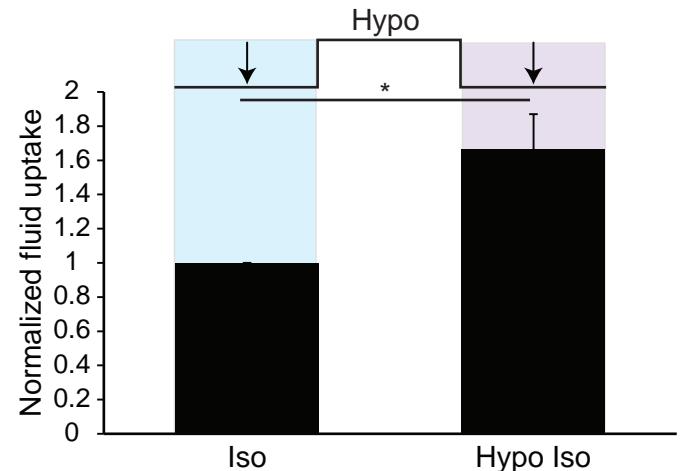
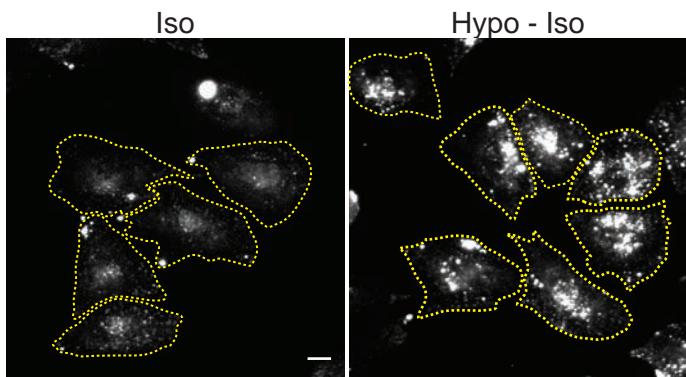
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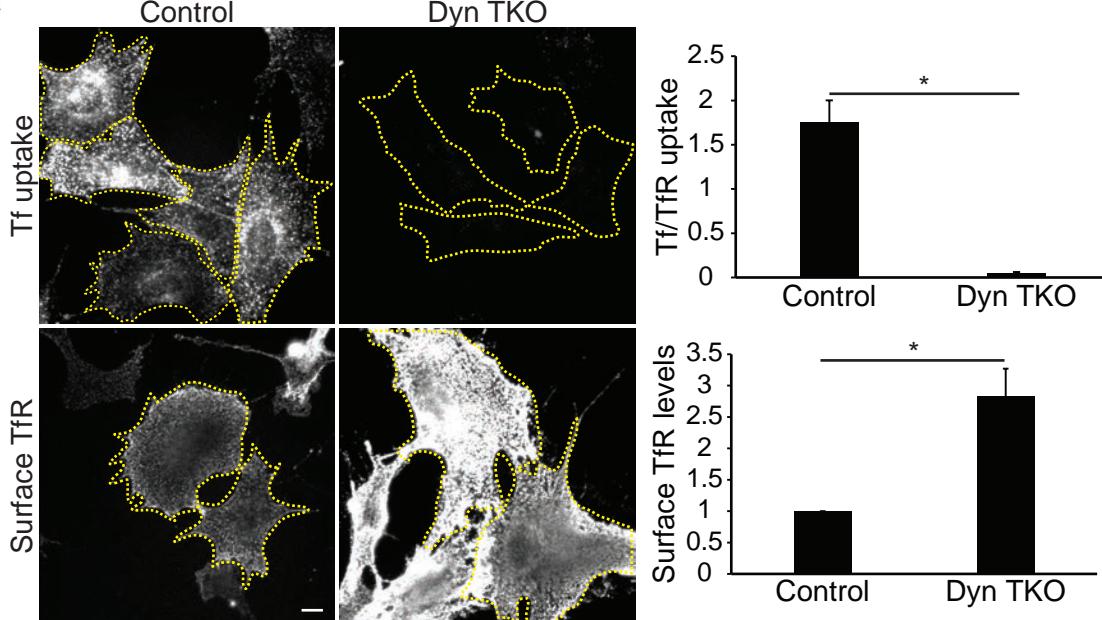
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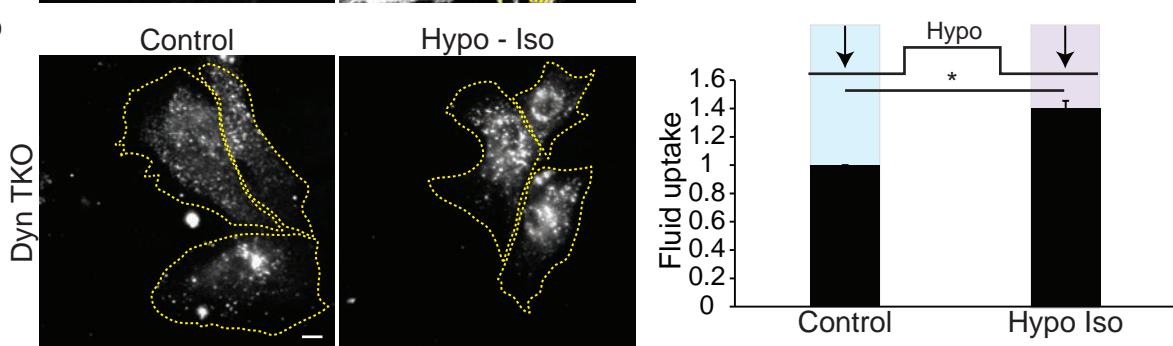
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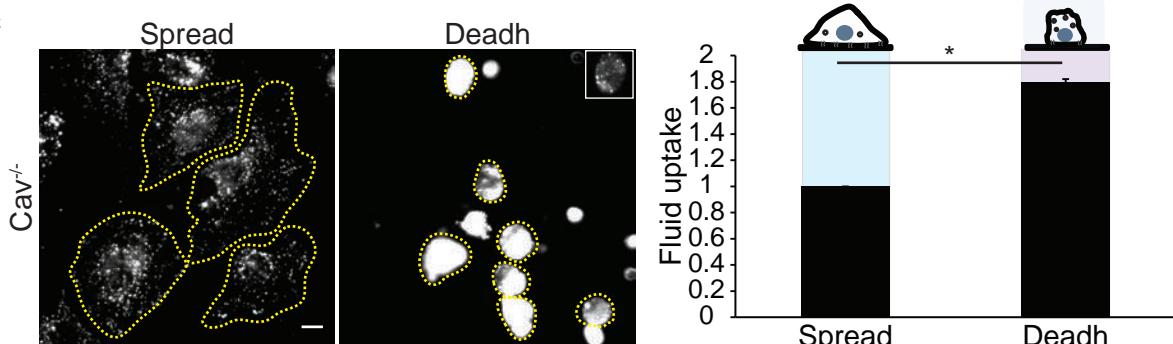
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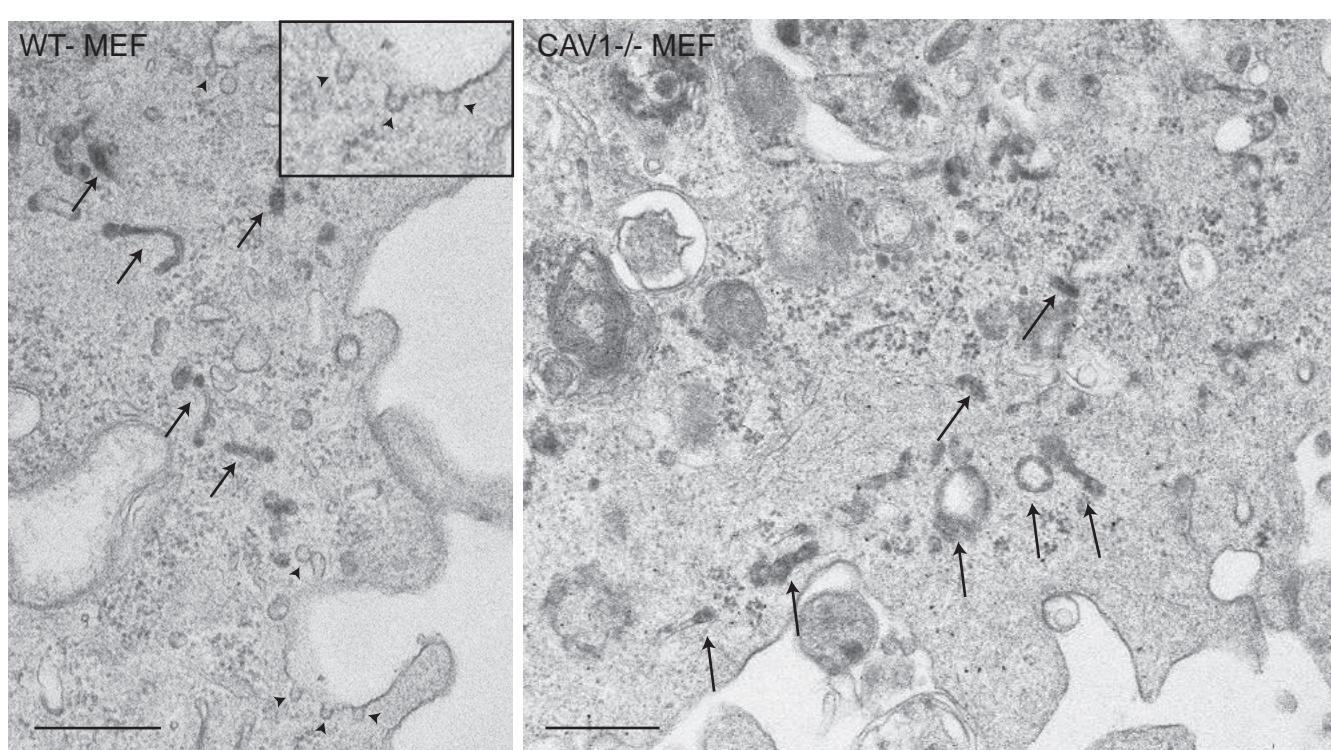
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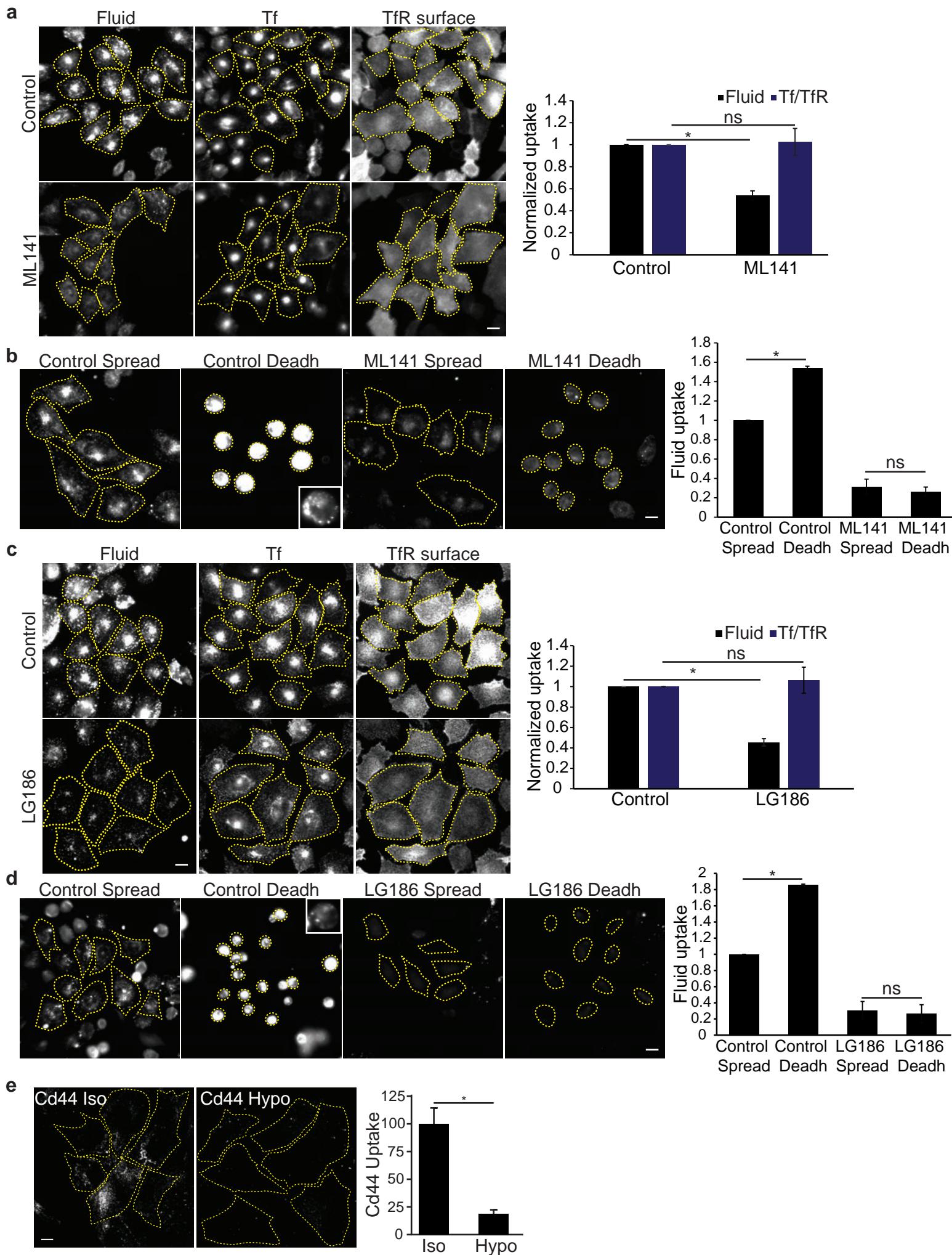
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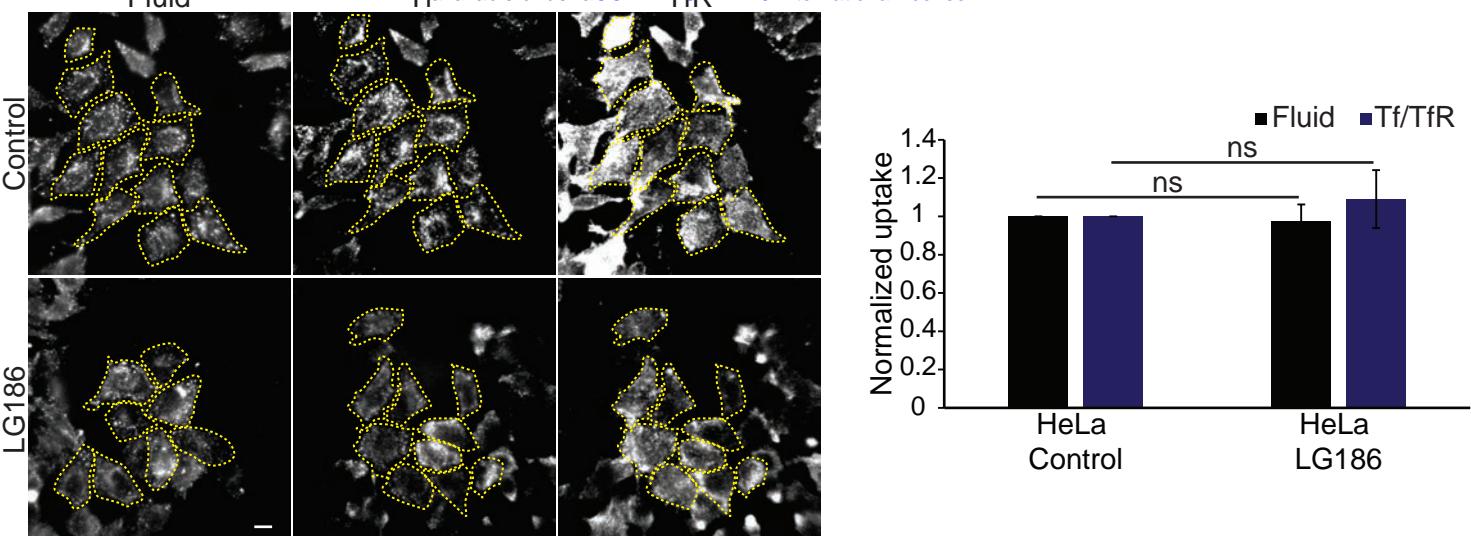
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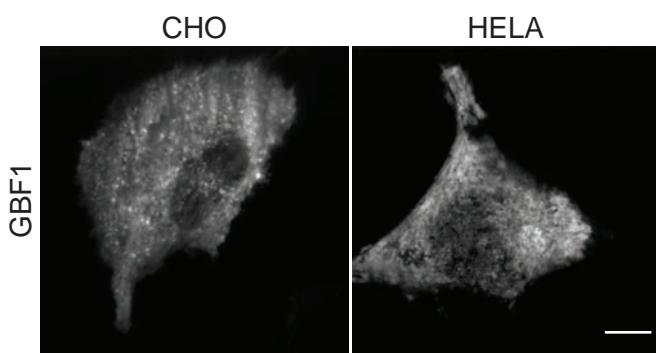
SUPPLEMENTARY FIGURE 2



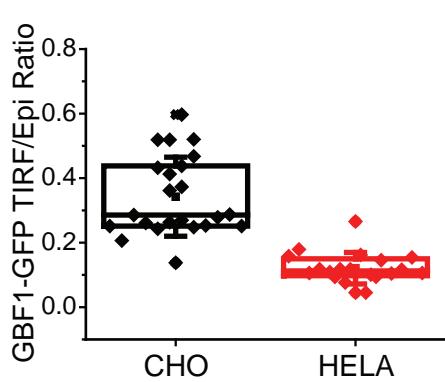
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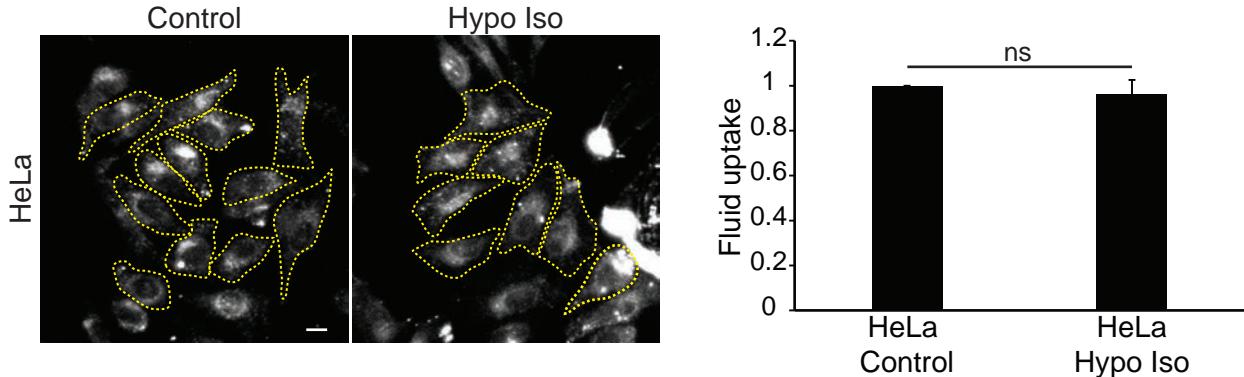
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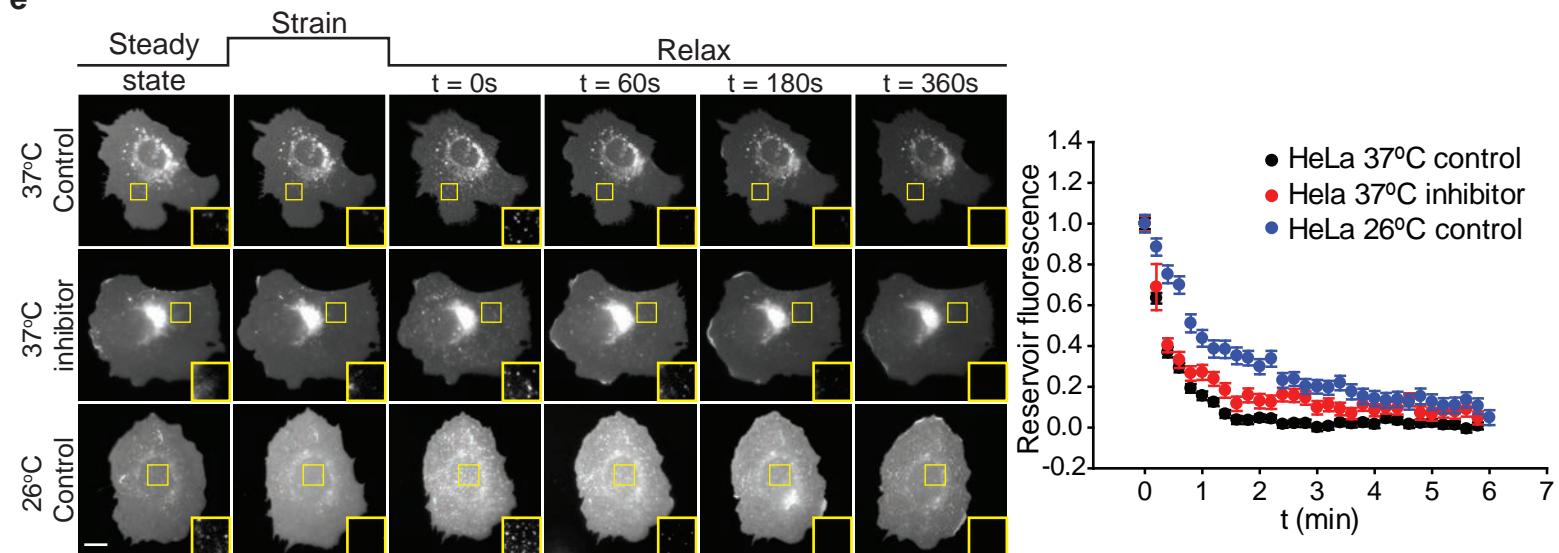
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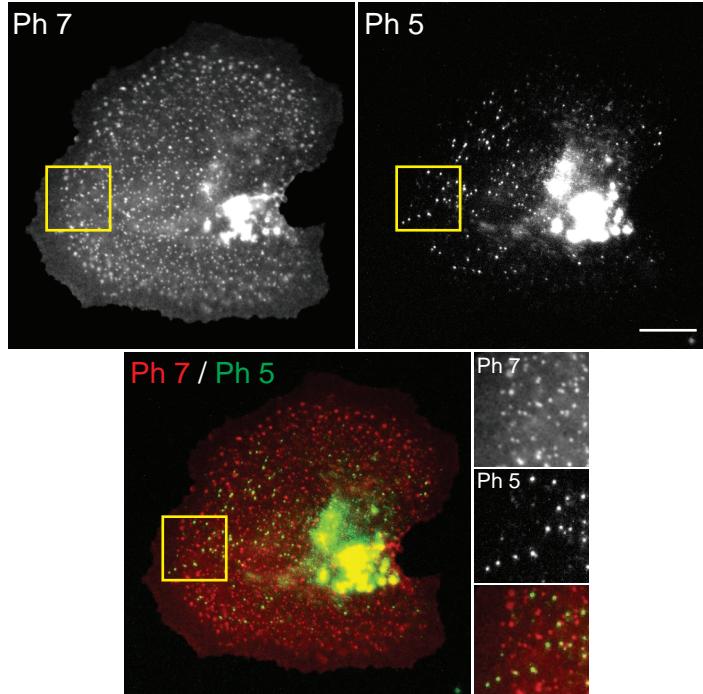
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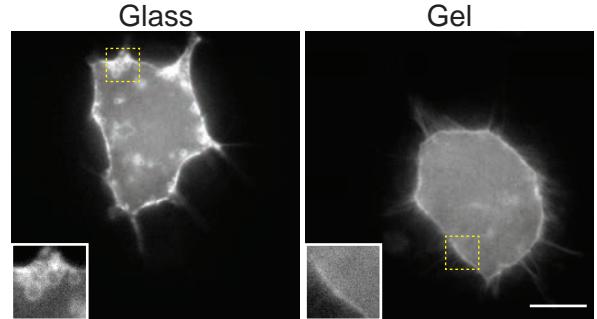
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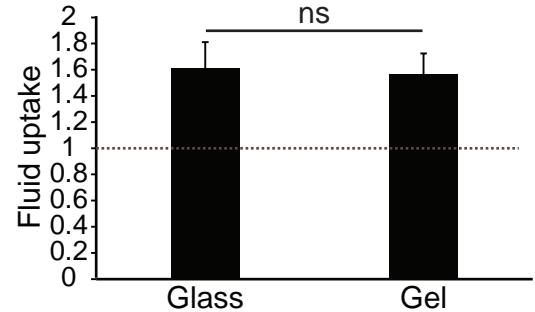
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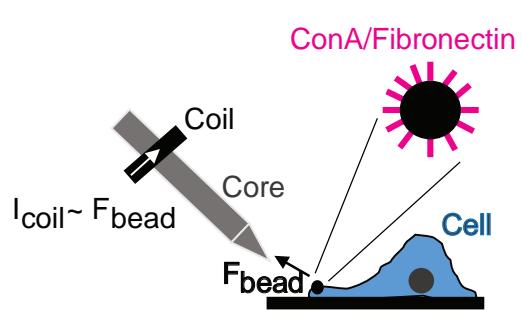
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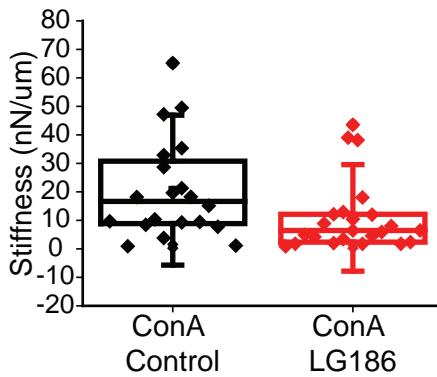
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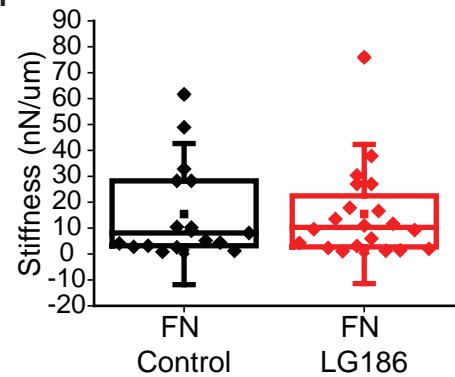
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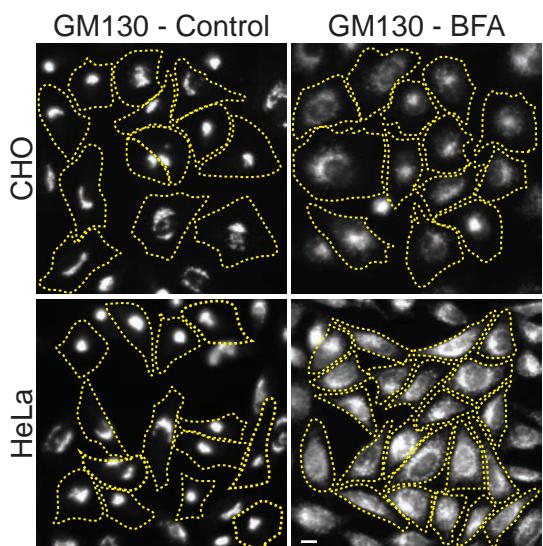
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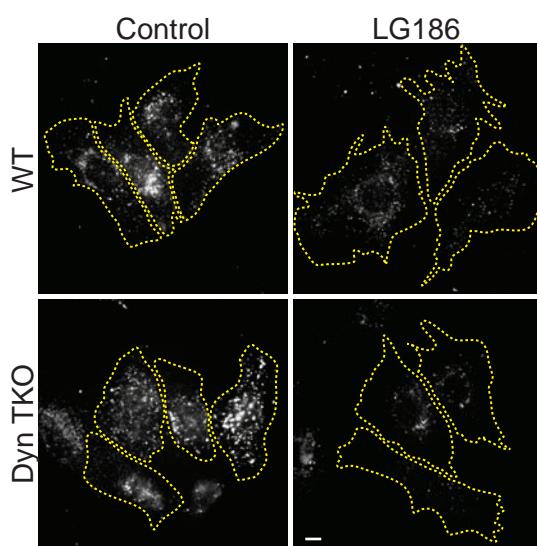
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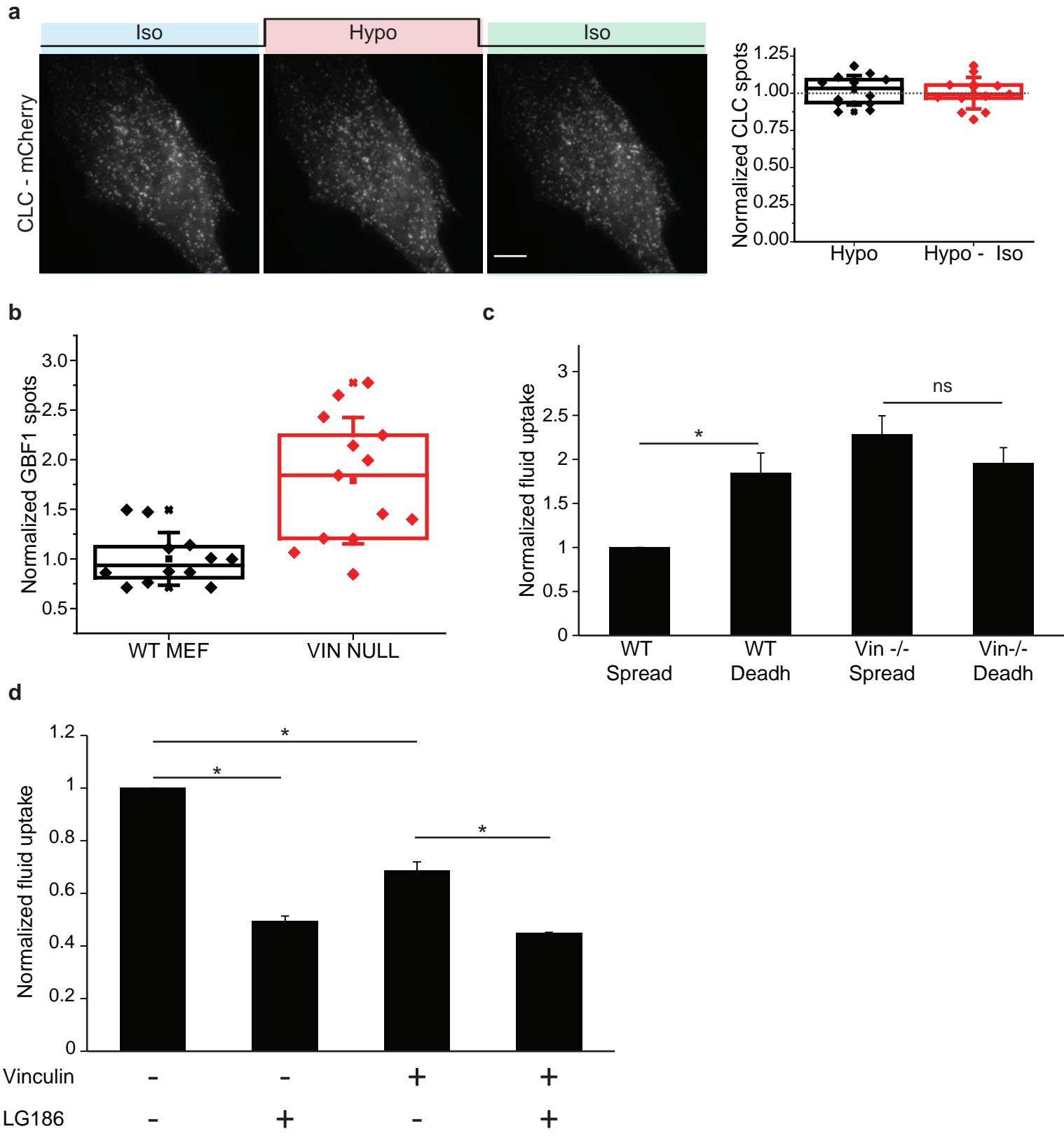


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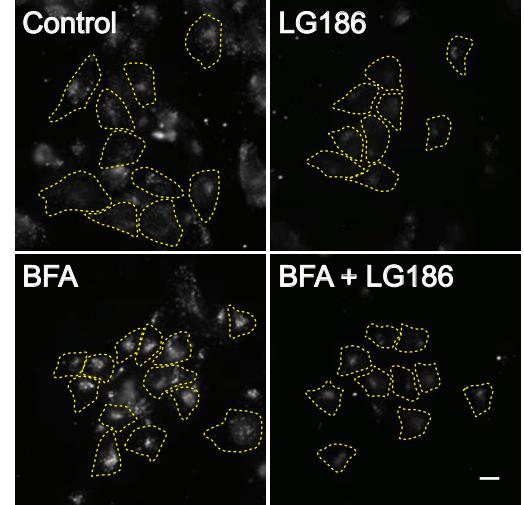
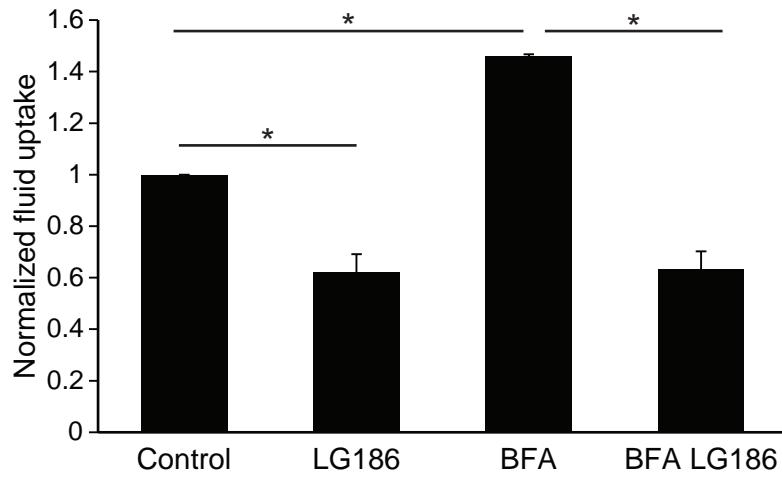
h





SUPPLEMENTARY FIGURE 6

a



b

